(12) INTERNATIONAL APP ION PUBLISHED UNDER THE PATENT ERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
27 September 2001 (27.09.2001)

PCT

(10) International Publication Number
WO 01/70993 A2

(51) International Patent Classification[7]: C12N 15/53, 9/02, 15/63, 5/10, C12P 21/00, G01N 33/50, 33/573, C12Q 1/68, 1/26, A61K 38/44

(21) International Application Number: PCT/CA01/00398

(22) International Filing Date: 26 March 2001 (26.03.2001)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
2,301,158 24 March 2000 (24.03.2000) CA

(71) Applicant *(for all designated States except US)*: **SCOTIA HOLDINGS PLC** [GB/GB]; 40 Town Hill, London, Greater London EC3N 4BB (GB).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **WINTHER, Michael, David** [CA/CA]; 10 Anderson Blvd., Kentville, Nova Scotia B4N 5G9 (CA). **SMITH, Heidi, Lynn** [CA/CA]; 20 Glenview Avenue, Kentville, Nova Scotia B4N 2R5 (CA). **ALLEN, Stephen, John** [CA/CA]; 9049 Commercial St., Suite 150, New Minas, Nova Scotia B4N 5A4 (CA). **PONTON, Andre** [CA/CA]; 15 Oakwood Drive, Kingston, Nova Scotia B0P 1R0 (CA). **DE ANTUENO, Roberto, Justo** [CA/CA]; 1520 Ashlee Dr., Coldbrook, Nova Scotia B4R 1A1 (CA).

(74) Agent: **HELLER, David, J.**; Ridout & Maybee, One Queen Street East, Suite 2400, Toronto, Ontario M5C 3B1 (CA).

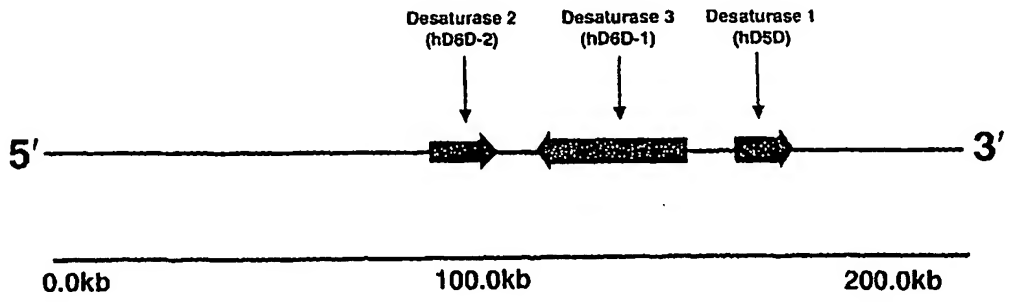
(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:
— *without international search report and to be republished upon receipt of that report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: POLYNUCLEOTIDES THAT CONTROL DELTA-6-DESATURASE GENES AND METHODS FOR IDENTIFYING COMPOUNDS FOR MODULATING DELTA-6-DESATURASE



(57) Abstract: The present invention relates to polynucleotides that control desaturase genes and to drug screening assays for identifying pharmaceutially active compounds for use in the treatment of diseases involving abnormal lipid metabolism including diabetic neuropathy, by utilizing fatty acid desaturase enzymes and the genes which encode them as targets for intervention. The drug screening method identifies nucleotides, proteins, compounds and/or other pharmacological agents, which effectively modulate the activity of desaturase enzymes or regulate the level of expression of the desaturase genes.

WO 01/70993 A2

metabolism disorder in a subject by detecting a germline alteration in a polynucleotide of the invention in the subject, wherein the polynucleotide encodes a mammalian delta-6-desaturasecomprising comparing the germline sequence of the polynucleotide from a tissue sample from the subject with the germline sequence of a wild-type of the polynucleotide, wherein an alteration in the germline sequence of the subject indicates the presence of or a predisposition to the lipid metabolism disorder. The method may be selected from the group consisting of immunoblotting, immunocytochemistry, enzyme-linked immunosorbent assay, DNA fingerprinting, in situ hybridization, polymerase chain reaction, reverse transcription polymerase chain reaction, radioimmunoassay, immunoradiometric assay and immunoenzymatic assay.

The invention further teaches a method for diagnosing the presence of or a predisposition for a lipid metabolic disorder in a subject, comprising comparing the polypeptide sequence of a control region of delta-6-desaturase from a tissue sample from the subject with the sequence of a wild-type of the delta-6-desaturase, wherein an alteration in the sequence of the subject as compared to the wild-type indicates the presence of or a predisposition to the lipid metabolic disorder.

The invention further teaches a method for identifying a compound which inhibits or promotes the activity of control regions of mammalian delta-6- and/or delta-5-desaturases, comprising the steps of: (a) selecting one or more host cells comprising the polynucleotides, wherein such host cells are heterogeneous to the polynucleotides; (b) cloning the host cells and separating the clones into a test group and a control group; (c) treating the test group using a compound; and (d) determining the relative quantities of expression products of operably linked polynucleotides to the control regions, as between the test group and the control group. Also taught is a composition for treating a lipid metabolism disorder comprising a compound identified by a method of the invention and a pharmaceutically acceptable carrier.

The invention also teaches a compound identified by the methods of the invention. The invention further teaches the use of a compound as the inventioned in the invention for treating a lipid metabolism disorder.

A host cell of the invention may be a spheroplast. The spheroplast may be a *Saccharomyces cerevisiae.*

The disorders of the invention may be selected from the group consisting of atopic eczema, mastalgia, rheumatoid arthritis, Sjögren's syndrome, gastrointestinal disorders, viral infections and postviral fatigue, pre-menstrual syndrome, endometriosis, cystic fibrosis, schizophrenia,

alcoholism, congenital liver disease, Alzheimer's syndrome, Crohn's disease, cardiovascular disease, cancer, diabetes and diabetic complications. The diabetic complication may be selected from the group consisting of diabetic neuropathy, nephropathy and retinopathy.

The compounds of the invention may be selected from the group consisting of small organic molecules, peptides, polypeptides, antisense molecules, oligonucleotides, polynucleotides, fatty acids and derivatives thereof.

The invention further teaches the use of a composition of the invention for treating a lipid metabolic disorders.

These and other advantages and features of novelty, which characterize the invention, are pointed out with particularity in the inventions annexed hereto and forming a part hereof. For a better understanding of the invention, its advantages, and objects obtained by its use, reference may be made to the accompanying drawings and descriptive matter, in which there is illustrated and described preferred embodiments of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

In the following description, the invention will be explained in detail with the aid of the accompanying figures, which illustrate preferred embodiments of the present invention and in which:

Figure 1 shows the nucleic acid sequence (SEQ ID NO:1) of the rD6D-1 control region;

Figure 2 shows the nucleic acid sequence (SEQ ID NO:2) of the hD6D-1 control region;

Figure 3 shows the nucleic acid sequence of the rD6D-1 coding portion of the fatty acid desaturase gene;

Figure 4 shows the nucleic acid sequence of the hD6D-1 coding portion of the fatty acid desaturase gene;

Figure 5 shows the amino acid sequences of the native rD6D-1 and C-terminal tagged enzymes, respectively;

or mammalian cells. More specifically, an embodiment of the present invention relates to a drug screening assay using transformed yeast as whole cells, spheroplasts, cell homogenates, organelles (e.g. microsomes, etc.) or purified enzyme to identify candidate agents that modulate the enzymatic activity of a mammalian D6D. In an embodiment of the present invention the host yeast *Saccharomyces cerevisiae,* strain INVSc1 (Invitrogen, CA), is transformed with the yeast expression vectors, pYES2 or pYES2/CT (Invitrogen), containing the mammalian D6D coding sequence. Yeast cells are selected for use in the present method because (1) they have not shown fatty acid delta-6-desaturase activity (Aki et al., 1999, *Biochem. Biophys. Res. Commun.*, 255: 575-579), (2) their transcription and translation processes are similar, if not identical, to processes that occur in mammalian cells, and (3) they are often more amenable to genetic manipulation than mammalian cells, and they grow much more rapidly (Guthrie C. and Fink G., 1991, *Meth. Enzymol.*, 194). Thus, yeast cells provide an excellent model for eukaryotic gene expression and for studying the modulation of mammalian D6D activity.

When a host cell, such as a yeast cell, is transformed with a DNA construct according to the present invention, it is utilized in assays to identify test components that modulate desaturase activity. Test components that modulate D6D activity are identified by (1) contacting the transformed host cell with the test component for a fixed period of time, and (2) determining the level of lipid metabolite (i.e. the level of product produced from substrate) or associated cofactors within the treated cells. This level of metabolite in one cell can then be compared to the level of metabolite in the absence of the test component. The difference between the levels of metabolite, if any, indicates whether the test component of interest modulates D6D activity. Furthermore, the magnitude of the level of lipid metabolite generated between the treated and untreated cells provides a relative indication of the strength of that compound(s) as a modulator of desaturase activity. Rat liver microsomes are used in conjunction with the preferred host system to corroborate the strength of that compound(s) as a modulator of desaturase activity.

A drug screening assay is also carried out using mammalian cells as host systems to observe the regulation of D6D gene expression and identify test components that modulate the expression of a reporter gene driven by D6D gene control regions or regulatory elements. ZR-75-1 or HepG2 cell lines are preferably used as the host systems, which are transfected with the reporter vectors, pCAT-3-Basic (Promega) or pGL3-Basic (Promega) containing the mammalian D6D control sequence.

When a preferred host cell line, such as ZR-75-1, is transfected with a reporter DNA construct according to the present invention, it is utilized in assays to identify test components that modulate the level of gene transcription via functionally active regulatory elements/oligonucleotide sequences.

Test components that alter the level of gene transcription can be identified by (1) contacting the transfected host cell with the test component for a fixed period of time, and (2) determining the level of gene expression (e.g. CAT activity) within the treated cells. This expression level is compared to that of the reporter gene in the absence of the compound(s). The difference between the levels of gene expression, if any, indicates whether the compound(s) of interest modifies the functionality of the DNA regulatory elements. Furthermore, the magnitude of the level of reporter product expressed between the treated and untreated cells provides a relative indication of the strength of that compound(s) as a modulator of the D6D gene transcription via transcriptional DNA regulatory elements.

In an embodiment, a high-throughput screening protocol is used to survey a large number of test compounds for their ability to modulate or regulate the transcription of a mammalian D6D gene through their effect on the desaturase control region. Accordingly, the design of the transcriptional system makes it possible to screen a large selection of components as potential therapeutic agents that alter D6D gene expression thereby increasing or decreasing tissue levels of a functional D6D enzyme, the physiological significance of which includes the normalization of lipid metabolites.

For the drug screening methods described herein, the host system may be a cell, tissue, organ, organism or any part thereof, which provides an environment or conditions that allow for, or enable, transcription and/or transcription followed by subsequent translation to yield a functional protein or polypeptide. Organisms would include animals such as mammals. In an embodiment of the invention, the drug screening methods are conducted in prokaryotic and eukaryotic cells. In embodiments of the invention, the eukaryotic cells include yeast cells and mammalian cells.

Potential antagonists include small organic molecules, peptides, polypeptides and antibodies that bind to a polynucleotide or polypeptide of the invention and thereby inhibit or extinguish its activity. Potential antagonists also may be small organic molecules, a peptide, a polypeptide such as a closely related protein or antibody that bind the same sites on a binding molecule, such as a binding molecule, without inducing delta-6-desaturase-induced activities, thereby preventing the action of delta-6-desaturase by interfering with substrate binding.

Potential antagonists include a small molecule, which bind to and occupy the binding site of the polypeptide thereby preventing binding to cellular binding molecules, such that normal biological activity is prevented. Examples of small molecules include but are not limited to small organic molecules, peptides or peptide-like molecules. Other potential antagonists include antisense molecules (see Okano et al., 1988, *EMBO J.*, 7: 3407-3412 for a description of these molecules).

Selective modulators may include, for example, antibodies and other proteins or peptides which specifically bind to the delta-6-desaturase or delta-6-desaturase nucleic acid, oligonucleotides which specifically bind to delta-6-desaturase (see Patent Cooperation Treaty International Publication No. WO93/05182 published Mar. 18, 1993) which describes methods for selecting oligonucleotides which selectively bind to target biomolecules) or delta-6-desaturase nucleic acid (e.g. antisense oligonucleotides) and other non-peptide natural or synthetic compounds which specifically bind to the delta-6-desaturase or delta-6-desaturase nucleic acid.

Targets for the development of selective modulators include, for example: (1) the regions of the delta-6-desaturase which contact other proteins and/or localize the delta-6-desaturase within a cell and (2) the regions of the delta-6-desaturase which bind substrate.

Thus, according to another aspect of the invention there is provided a drug screening method for identifying nucleotides, proteins, compounds and/or pharmacological agents that effectively modulate the activity of fatty acid desaturase enzymes and hence, fatty acid profiles. The method comprises (1) producing a nucleic acid construct having a promoter region, which is preferably induced, a nucleic acid sequence encoding a functional fatty acid desaturase enzyme, whereby the promoter region is operably associated with the nucleic acid sequence, and a termination sequence, all of which are introduced into a cell or cell lysate using an expression vector containing the nucleic acid construct, (2) contacting the cell or cell lysate with a test component, (3) evaluating the enzymatic activity of a desaturase polypeptide encoded by the nucleic acid sequence by assaying for a measurable difference in the level of lipid metabolite as an indicator of the ability of the test component to modulate fatty acid desaturase enzyme activity, and (4) selecting those components which exhibit such activity. The known substrate for the fatty acid desaturase may optionally be exogenously supplied to the cell or cell lysate.

Accordingly, the host system is transformed/transfected by the nucleic acid construct containing the nucleic acid sequence of the fatty acid desaturase gene such that the promoter region and the termination region are operable and can, therefore, be used to achieve high level expression of a functionally active desaturase enzyme. A test component which increases or decreases desaturase enzyme activity is an enhancer or inhibitor, respectively. Consequently, defined test components can be used as a basis for the formulation or innovation of therapeutic agents to treat disease related to the level of active and regulated fatty acid desaturase enzymes in tissue.

A microsomal host system may be achieved by transforming/transfecting the host system with the nucleic acid construct containing the coding sequence for a functional mammalian desaturase

DERWENT-ACC-NO: 2001-611507

DERWENT-WEEK: 200429

TITLE: Nucleic acid encoding delta-6-desaturase gene useful for treating atopic eczema, mastalgia, rheumatoid arthritis, Sjogren's syndrome, gastrointestinal disorders, viral infections and post viral fatigue

INVENTOR: ALLEN, S J; DE ANTUENO, R J ; PONTON, A ; SMITH, H L ; WINTHER, M D ; ANTUENO, R J D ; SMITH-KING, H L

PRIORITY-DATA: 2000CA-2301158 (March 24, 2000)

PATENT-FAMILY:

PUB-NO	PUB-DATE	LANGUAGE	PAGES	MAIN-IPC
WO 200170993 A2	September 27, 2001	E	164	C12N 015/53
CA 2301158 A1	September 24, 2001	E	000	C12Q 001/00
AU 200144002 A	October 3, 2001	N/A	000	C12N 015/53
EP 1268810 A2	January 2, 2003	E	000	C12N 015/53
US 20040053234 A1	March 18, 2004	N/A	000	C12Q 001/68

DESIGNATED-STATES: AE AG AL AM AT AU AZ BA BB BG BR BY BZ CA CH CN CR CU CZ DE
DK DM DZ EE ES FI GB GD GE GH GM HR HU ID IL IN IS JP KE KG KP KR KZ LC LK LR
LS LT LU LV MA MD MG MK MN MW MX MZ NO NZ PL PT RO RU SD SE SG SI SK SL TJ TM
TR TT TZ UA UG US UZ VN YU ZA ZW AT BE CH CY DE DK EA ES FI FR GB GH GM GR IE
IT KE LS LU MC MW MZ NL OA PT SD SE SL SZ TR TZ UG ZW AL AT BE CH CY DE DK ES
FI FR GB GR IE IT LI LT LU LV MC MK NL PT RO SE SI TR

INT-CL (IPC): A61K038/44, C07H021/04 , C12N005/10 , C12N009/02 ,

WO 01/70993, pub. 9/27/01

US 20040053234A1

(19) **United States**

(12) **Patent Application Publication** (10) **Pub. No.: US 2004/0053234 A1**

Winther et al. (43) **Pub. Date: Mar. 18, 2004**

(54) **POLYNUCLETIDES THAT CONTROL DELTA-6 DESATURASE GENES AND METHODS FOR IDENTIFYING COMPOUNDS FOR MODULATING DELTA-6 DESATURASE**

(76) Inventors: **Michael David Winther**, Vancouver (CA); **Heidi Lynn Smith-King**, Hantsport (CA); **Andre Ponton**, St. Hubert (CA); **Roberto Justo De Antueno**, Coldbrook (CA); **Stephen John Allen**, Coldbrook (CA)

Correspondence Address:
David J Heller
Ridout & Maybee
Suite 2400
One Queen Street East
Toronto, ON M5C 3B1 (CA)

(21) Appl. No.: **10/239,652**

(22) PCT Filed: **Mar. 26, 2001**

(86) PCT No.: **PCT/CA01/00398**

(30) **Foreign Application Priority Data**

Mar. 24, 2000 (CA) 2301158

Publication Classification

(51) **Int. Cl.**[7] **C12Q 1/68**; C07H 21/04; C12N 9/04; A61K 38/44

(52) **U.S. Cl.** **435/6**; 435/69.1; 435/190; 435/320.1; 435/325; 536/23.2; 424/94.4

(57) **ABSTRACT**

The present invention relates to polynucleotides that control desaturase genes and to drug screening assays for identifying pharmaceutially active compounds for use in the treatment of diseases involving abnormal lipid metabolism including diabetic neuropathy, by utilizing fatty acid desaturase enzymes and the genes which encode them as targets for intervention. The drug screening method identifies nucleotides, proteins, compounds and/or other pharmacological agents, which effectively modulate the activity of desaturase enzymes or regulate the level of expression of the desaturase genes.

POLYNUCLETIDES THAT CONTROL DELTA-6 DESATURASE GENES AND METHODS FOR IDENTIFYING COMPOUNDS FOR MODULATING DELTA-6 DESATURASE

FIELD OF THE INVENTION

[0001] This invention relates to the identification of nucleotides, proteins, compounds and/or pharmacological agents that either inhibit or enhance the activity of fatty acid delta-6-desaturase enzymes involved in lipid metabolism and/or effectively regulate the level of expression of the delta-6-desaturase genes, and to compounds so identified.

BACKGROUND OF THE INVENTION

[0002] The first committed step in the biosynthetic pathway for polyunsaturated fatty acids (PUFAs) is catalyzed by an enzyme known as delta-6-desaturase (D6D) which catalyzes the synthesis of GLA from LA. This occurs in the n-6 metabolic pathway. In addition, D6D also converts ALA into stearidonic acid (SDA) in the n-3 metabolic pathway. GLA is subsequently converted as a substrate to DGLA through an elongation process, which is then converted to AA through desaturation by a different desaturase enzyme known as delta-5-desaturase (D5D). AA and DGLA are essential precursors of various important eicosanoids. These PUFAs are subsequently incorporated into membrane phospholipids and used for eicosanoid biosynthesis.

[0003] The amino acid and nucleotide sequences for human delta-6-desaturase have been found, (Cho et al., 1999a, *J. Biol. Chem.,* 274: 471-477), and the rat delta-6-desaturase characterized to some extent (Aki et al., 1999, *Biochem. Biophys. Res. Commun.,* 255: 575-579).

[0004] It has been reported that endogenous GLA formation is impaired in a number of disease states and subsequent administration of GLA has therapeutic effect (Horrobin D. F., 1990, *Rev. Contemp. Pharmacother,* 1: 1-45 and Horrobin D. F., 1992, *Prog. Lipid Res.,* 31: 163-194). The types of diseases which have been studied include the following: atopic eczema, diabetic neuropathy, mastalgia, rheumatoid arthritis, Sjogren's syndrome, gastrointestinal disorders, viral infections and postviral fatigue, endometriosis, schizophrenia, alcoholism, Alzheimer's syndrome, cardiovascular disease, renal disease, cancer and liver disease. Also, in several other human diseases (e.g. cystic fibrosis, Cohn's disease and congenital liver disease) abnormal patterns of PUFAs attributable to insufficient dietary LA or to altered metabolism such as diminished capabilities involving desaturation or chain elongation have been described (Cook H. W., 1996, *Fatty Acid Desaturation and Chain Elongation in Eukaryotes: in Biochemistry of Lipids, Lipoproteins and Membranes*, Vance D. E. and Vance J. E. (eds.) Elsevier, Amsterdam, pp.150).

[0005] Furthermore, severe effects observed in experimental animals and humans in the absence of dietary essential fatty acids include a dramatic decrease in weight, dermatosis and increased permeability to water, enlarged kidneys and reduced adrenal and thyroid glands, cholesterol accumulation and altered fatty acyl composition in many tissues, impaired reproduction and ultimate death (Sinclair H. M., 1984, *Hum. Nutr. Clin. Nutr.,* 38: 245-260).

[0006] The pathophysiology of diabetic peripheral neuropathy appears to be associated with the abnormal metabolism of essential fatty acids (Julu P., 1997, in *Essential Fatty Acids and Eicosanoids*, pp.168-175). This abnormal or altered lipid metabolism is reflected in the lack of incorporation of n-6 fatty acids in membrane phospholipids (Coste et al., 1999, *J. Nutr. Biochem.,* 10: 411-420). Evidence from experimental diabetes studies in animals indicates that the formation of fatty acids by the desaturation and elongation systems is impaired which may lead to an abnormal polyunsaturated fatty acid metabolism. Based on these findings, it has been proposed that if the rate-limiting step of the reaction involving the delta-6-desaturation of LA is bypassed by way of administration of the product, GLA, it may be possible to control or reduce some of the pathophysiological symptoms associated with diabetic neuropathy (Cotter M. A. and Cameron N. E., 1997, *Diabetic Neuropathy*, Marius Press, Carnforth, U.K., pp. 97-119). Oils containing substantial amounts of n-6 fatty acids, in particular GLA, have prevented nerve conduction velocity deficits. Indeed, GLA treatment in diabetic rats prevented nerve conduction velocity deficits probably by the repletion of a discrete pool of arachidonic acid in phospholipids which is critical for normal nerve function (Kuruvilla et al., 1998, *Prostaglandins Leukot. Essent. Fatty Acids,* 59: 196-202 and Coste et al., 1999, *J. Nutr. Biochem.,* 10: 411-420). The precise mechanism by which GLA brings about these improvements has not yet been established.

[0007] In human diabetics, trends observed in results derived from several clinical trials resemble those found in animal models. For example, multicenter clinical trials have shown promising results with GLA treatment in that the administration of GLA partially normalizes nerve conduction velocity and other neurophysiological parameters, thereby reducing symptoms of diabetic neuropathy (Keen et al. 1993, *Diabetes Care,* 16: 8-15). Recent studies have also shown that the therapeutic effect of GLA in diabetes can be enhanced by the addition of other compounds that affect lipid oxidation (Tomlinson D. R, 1998, *Diabetes and Metabolism* (Paris), 24 (suppl) 3: 79-83). Data compiled from different laboratory studies suggest that GLA is the main active n-6 PUFA for treatment of diabetic neuropathy.

[0008] However, fatty acids of the n-3 family are not as effective when tested in animal models of diabetes. Furthermore, it has been shown that the administration of n-3 fatty acids in combination with GLA can actually reduce the incorporation of GLA and subsequently impair the effectiveness of GLA in its ability to reverse nerve conduction velocity deficits (Dines et al., 1993, *Diabetologia,* 30: 1132-1138).

[0009] U.S. Pat. Nos. 4,806,569 and 4,826,877 teach that the conversion of LA and ALA is deficient in certain disease conditions including diabetes. The deficiency has been identified as a lack of activity of the first enzyme in the pathway, being D6D. As a consequence, diabetic patients have a higher concentration of LA with a concomitant reduction in the AA concentration. These results have been confirmed and expanded upon with the present inventors' work

[0010] Eczema is a superficial inflammation of the skin, which affects both the dermis and the epidermis. The role of polyunsaturated fatty acids in the treatment of atopic eczema was initially proposed (Hansen A. E., 1933, *Proc. Soc. Exp. Biol. Med.* 31: 160-161) after it was discovered that patients who suffered from eczema also had elevated serum levels of

LA, but reduced levels of delta-6-desaturase products, such as GLA. These findings were later supported and expanded by other studies in which eczema patients showed low levels of serum arachidonic acid (Manku et al., 1984, *Br. J. Dermatol,* 110: 643-680). A placebo-controlled clinical study confirmed the therapeutic usefulness of GLA supplementation in atopic eczema (Wright et al., 1982, *Lancet,* 2: 1120-1122). However, it has subsequently been reported that while providing oils rich in GLA produces a symptomatic improvement for atopic eczema, the treatment does not change the underlying disease state (Chapman & Hall ed., 1992, *Unsaturated Fatty Acids: Nutritional and Physiological Significance*, British Nutrition Foundation, London, pp. 175).

[0011] The cytotoxic effects of GLA and EPA have been shown to be selective for cancer cells without affecting normal cells in vitro (Begin et al., 1986, *J. Nat. Cancer Inst.,* 77: 1053-1062 and Vartak et al., 1997, *Br. J. Cancer,* 77: 1612-1620). In addition, through elongation and desaturation steps, GLA and EPA are precursor molecules of other PUFAs of relevant importance in oncology, such as dihomo-gamma-linolenic acid (DGLA) and docosahexaenoic acid (DHA). In this regard, several studies have shown that treatment of malignant cells with EPA, GLA and/or their metabolites leads to cell cycle arrest, induction of apoptosis, inhibition of mitosis (Seegers et al., 1997, *Prostaglandins Leukot. Essent. Fatty Acids,* 56: 271-280 and Lai et al., 1996, *Br. J. Cancer,* 74: 1375-1383) and cell proliferation (Calviello et al., 1998, *Int. J. Cancer,* 75: 699-705), anti-metastatic mechanisms, regulation of a metastasis-suppressor gene and occluding expression (Jiang et al, 1998a, *Br. J. Cancer,* 77: 731-738 and Jiang et al., 1998b, *Biochem. Biophys. Res. Commun.,* 244: 414-420), reduction of tumor-endothelium adhesion, improvement of gap junction communications of the endothelium (Jiang et al., 1997, *Prostaglandins Leukot. Essent. Fatty Acids,* 56: 307-316), inhibition of urokinases (du Toit et al., 1994, *Prostaglandins Leukot. Essent. Fatty Acids,* 51: 121-124) and induction of E-cadherin (Jiang et al., 1995a, *Br. J Cancer,* 71: 5043-5048), reduction of the effects of growth factors on cancer cells (Jiang et al., 1995b, *Br. J. Cancer,* 71: 744-752), reversion of multi-drug resistance (Weber et al., 1994, *J. Nat. Cancer Inst.,* 86: 638-639), and increase of the cytotoxic effects of chemotherapeutic agents (Plumb et al., 1993, *Br. J. Cancer,* 67: 728-733 and Anderson et al., 1998, *Anticancer Res.,* 18: 791-800). In particular, EPA has been reported to significantly inhibit the growth of human pancreatic cancer cell lines in vitro (Falconer et al., 1994, *Br. J. Cancer,* 69: 826-832) and down-regulate the acute-phase response in patients with pancreatic cancer cachexia (Wigmore et al., 1997, *Clin. Sci.,* 92: 215-221). It has also been shown that following exposure to GLA or EPA, malignant cells generate much higher levels of potentially cytotoxic superoxide radicals and lipid peroxidation products (Takeda et al., 1993, *Anticancer Res.,* 13: 193-200).

[0012] In view of the beneficial effects of GLA, and the other essential products of D6D, there is a need for drug screening methods which identify test components which will modulate fatty acid desaturase activity or the level of desaturase gene expression for their subsequent utilization in the treatment and/or prevention of certain pathological disorders associated with abnormal lipid metabolism.

SUMMARY OF INVENTION

[0013] The present invention is directed to mammalian fatty acid desaturase enzymes and the use of their nucleic acid and amino acid sequences in expression vectors and host systems for drug screening methods. Test components identified through these methods can be used as a basis for the formulation or innovation of therapeutic drugs, or as lead compounds to design or search for other drugs.

[0014] The invention teaches (1) the isolation, cloning and identification of the control region (i.e. promoter and other regulatory elements) of both a human and a rat fatty acid desaturase gene and (2) the use of the desaturase gene control region in drug screening methods to identify test components which can effectively modulate desaturase gene expression. The present invention incorporates the knowledge that the particular genetic elements, which are responsible for controlling desaturase gene expression, can be isolated independently of the desaturase gene encoding region (i.e. amino acid coding sequences) and, therefore, be employed to assay for agents that modulate desaturase gene expression,

[0015] The invention thus provides an isolated polynucleotide segment, comprising a polynucleotide sequence which is selected from the group consisting of: (a) a sequence comprising SEQ ID NO: 1; (b) a sequence comprising SEQ ID NO: 2; (c) a sequence which is at least 80% homologous with a sequence of any of (a) to (b); (d) a sequence which is at least 90% homologous with a sequence of any of (a) to (b); (e) a sequence which is at least 95% homologous with a sequence of any of (a) to (b); (f) a sequence which is at least 98% homologous with a sequence of any of (a) to (b); (g) a sequence which is at least 99% homologous with a sequence of any of (a) to (b); and; (h) a sequence which hybridizes to any of (a) to (g) under stringent conditions.

[0016] The invention also teaches an isolated polynucleotide segment of the invention, wherein the isolated polynucleotide segment is genomic DNA. The invention teaches a vector comprising a polynucleotide segment of the invention in a suitable vector. Also provided is a host cell comprising a polynucleotide segment of the invention in a host cell which is heterogeneous to the segment.

[0017] The invention provides a method for producing a polypeptide encoded by a gene operably linked to a polynucleotide segment of the invention comprising the step of culturing the host cell of the invention under conditions sufficient for the production of the polypeptide.

[0018] The invention includes an isolated polynucleotide fragment selected from the group consisting of: (a) a sequence having at least 15 sequential bases of nucleotides of a segment of the invention; (b) a sequence having at least 30 sequential bases of nucleotides of a segment of the invention ; and (c) a sequence having at least 50 sequential bases of nucleotides of a segment of the invention.

[0019] Also taught is a vector comprising a polynucleotide segment of the invention contained in a vector which is heterogeneous to the segment. Also taught is an isolated polynucleotide segment, comprising a polynucleotide sequence which retains substantially the same biological function or activity as the polynucleotide encoded by a segment of the invention.

[0020] The invention teaches a method for identifying a compound which inhibits or promotes the activity of a polynucleotide segment of the invention, comprising the steps of: (a) selecting a control animal having the segment and a test animal having the segment; (b) treating the test animal using a compound; and, (c) determining the relative quantity of an expression product of an operably linked polynucleotide to the segment, as between the control animal and the test animal. The animals may be mammals. The mammals may be rats.

[0021] The invention further teaches a method for identifying a compound which inhibits or promotes the activity of a polynucleotide segment of the invention, comprising the steps of: (a) selecting a host cell of the invention; (b) cloning the host cell and separating the clones into a test group and a control group; (c) treating the test group using a compound; and (d) determining the relative quantity of an expression product of a polynucleotide operably linked to the polynucleotide segment, as between the test group and the control group.

[0022] The invention teaches a method for identifying a compound which inhibits or promotes the activity of a polynucleotide segment of the invention comprising the steps of: (a) selecting a test group having a host cell of the invention a part thereof or an isolated polynucleotide thereof and a control group; (b) treating the test group using a compound; and (c) determining the relative quantity of an expression product of an operably linked polynucleotide to the segment, as between the test group and the control group.

[0023] Also taught is a composition for treating a lipid metabolism disorder comprising a compound which modulates a segment according to the invention and a pharmaceutically acceptable carrier.

[0024] The invention further teaches a method for diagnosing the presence of or a predisposition for a lipid metabolic disorder in a subject by detecting a germline alteration in a segment of the invention in the subject, comprising comparing the germline sequence of a segment of the invention from a tissue sample from the subject with the germline sequence of a wild-type of the segment, wherein an alteration in the germline sequence of the subject indicates the presence of or a predisposition to the lipid metabolic disorder. The comparing may be performed by a method selected from the group consisting of immunoblotting, immunocytochemistry, enzyme-linked immunosorbent assay, DNA fingerprinting, in situ hybridization, polymerase chain reaction, reverse transcription polymerase chain reaction, radioimmunoassay, immunoradiometric assay and immunoenzymatic assay.

[0025] The invention further teaches a method for identifying a compound which inhibits or promotes the activity of a polynucleotide, wherein the polynucleotide encodes a mammalian delta-6-desaturase, comprising the steps of (a) selecting a control animal having the polynucleotide and a test animal having the polynucleotide; (b) treating the test animal using a compound; and, (c) determining the relative quantity of an expression product of the polynucleotide, as between the control animal and the test animal.

[0026] The invention teaches a method for identifying a compound which inhibits or promotes the activity of a mammalian delta-6-desaturase, comprising the steps of: (a) selecting a host cell of the invention; (b) cloning the host cell and separating the clones into a test group and a control group; (c) treating the test group using a compound; and (d) determining the relative quantity of an expression product of an expression polynucleotide operably linked to the polynucleotide segment, as between the test group and the control group.

[0027] The invention teaches a method for identifying a compound which inhibits or promotes the activity of a mammalian delta-6-desaturase, comprising the steps of: (a) selecting a test group haying a host cell of the invention a part thereof or an isolated polynucleotide thereof and a control group; (b) treating the test group using a compound; and (c) determining the relative quantity or relative activity of a product of the polynucleotide segment or of the polynucleotide segment, as between the test group and the control group.

[0028] The invention teaches a method for identifying a compound which inhibits or promotes the activity of a mammalian delta-6-desaturase, comprising the steps of: (a) selecting a control animal having a polypeptide segment of the invention and a test animal having the polypeptide segment; (b) treating the test animal using a compound; (c) determining the relative quantity or relative activity of an expression product of the polypeptide segment or of the the polypeptide segment, as between the control animal and the test animal.

[0029] The relative activity of the expression product may be determined by assaying for a conversion of 18:2n6 to 22:5n6. The relative activity of the expression product may be determined by assaying for a conversion of 18:3n3 to 22:6n3. The relative activity of the expression product may be determined by assaying for a conversion of 16:0 to 22:4n9.

[0030] The invention further teaches a use of a method according to the invention for identifying a modulator that modulates lipid metabolism disorders.

[0031] The invention also teaches a composition for treating a lipid metabolism disorder comprising a compound identified by any one of the methods of the invention and a pharmaceutically acceptable carrier.

[0032] The invention teaches a method for diagnosing the presence of or a predisposition for a lipid metabolism disorder in a subject by detecting a germline alteration in a polynucleotide of the invention in the subject, wherein the polynucleotide encodes a mammalian delta-6-desaturasecomprising comparing the germline sequence of the polynucleotide from a tissue sample from the subject with the germline sequence of a wild-type of the polynucleotide, wherein an alteration in the germline sequence of the subject indicates the presence of or a predisposition to the lipid metabolism disorder. The method may be selected from the group consisting of immunoblotting, immunocytochemistry, enzyme-linked immunosorbent assay, DNA fingerprinting, in situ hybridization, polymerase chain reaction, reverse transcription polymerase chain reaction, radioimmunoassay, immunoradiometric assay and immunoenzymatic assay.

[0033] The invention further teaches a method for diagnosing the presence of or a predisposition for a lipid metabolic disorder in a subject, comprising comparing the polypeptide sequence of a control region of delta-6desatu-

rase from a tissue sample from the subject with the sequence of a wild-type of the delta-6desaturase, wherein an alteration in the sequence of the subject as compared to the wild-type indicates the presence of or a predisposition to the lipid metabolic disorder.

[0034] The invention further teaches a method for identifying a compound which inhibits or promotes the activity of control regions of mammalian delta-6- and/or delta-5-desaturases, comprising the steps of. (a) selecting one or more host cells comprising the polynucleotides, wherein such host cells are heterogeneous to the polynucleotides; (b) cloning the host cells and separating the clones into a test group and a control group; (c) treating the test group using a compound; and (d) determining the relative quantities of expression products of operably linked polynucleotides to the control regions, as between the test group and the control group. Also taught is a composition for treating a lipid metabolism disorder comprising a compound identified by a method of the invention and a pharmaceutically acceptable carrier.

[0035] The invention also teaches a compound identified by the methods of the invention. The invention further teaches the use of a compound as the inventioned in the invention for treating a lipid metabolism disorder.

[0036] A host cell of the invention may be a spheroplast. The spheroplast may be a *Saccharomyces cerevisiae*.

[0037] The disorders of the invention may be selected from the group consisting of atopic eczema, mastalgia, rheumatoid arthritis, Sjögren's syndrome, gastrointestinal disorders, viral infections and postviral fatigue, pre-menstrual syndrome, endometriosis, cystic fibrosis, schizophrenia, alcoholism, congenital liver disease, Alzheimer's syndrome, Crohn's disease, cardiovascular disease, cancer, diabetes and diabetic complications. The diabetic complication may be selected from the group consisting of diabetic neuropathy, nephropathy and retinopathy.

[0038] The compounds of the invention may be selected from the group consisting of small organic molecules, peptides, polypeptides, antisense molecules, oligonucleotides, polynucleotides, fatty acids and derivatives thereof.

[0039] The invention further teaches the use of a composition of the invention for treating a lipid metabolic disorders.

[0040] These and other advantages and features of novelty, which characterize the invention, are pointed out with particularity in the inventions annexed hereto and forming a part hereof. For a better understanding of the invention, its advantages, and objects obtained by its use, reference may be made to the accompanying drawings and descriptive matter, in which there is illustrated and described preferred embodiments of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

[0041] In the following description, the invention will be explained in detail with the aid of the accompanying figures, which illustrate preferred embodiments of the present invention and in which:

[0042] **FIG. 1** shows the nucleic acid sequence (SEQ ID NO:1) of the rD6D-1 control region;

[0043] **FIG. 2** shows the nucleic acid sequence (SEQ ID NO:2) of the hD6D-1 control region;

[0044] **FIG. 3** shows the nucleic acid sequence of the rD6D-1 coding portion of the fatty acid desaturase gene;

[0045] **FIG. 4** shows the nucleic acid sequence of the hD6D-1 coding portion of the fatty acid desaturase gene;

[0046] **FIG. 5** shows the amino acid sequences of the native rD6D-1 and C-terminal tagged enzymes, respectively;

[0047] **FIG. 6** shows the amino acid sequences of the native hD6D-1 and C-terminal tagged enzymes, respectively,

[0048] **FIG. 7** shows the relative locations of the human "desaturase 1" (hD5D), "desaturase 2" (hD6D-2) and "desaturase 3" (hD6D1) genes on a segment of DNA from chromosome 11;

[0049] **FIG. 8** shows alternative splicing sites for exon 1 of the hD5D gene. "CRE" means cAMP responsive element.

[0050] **FIG. 9** shows the genomic exon-intron organization of hD5D, hD6D-2 and hD6D-1;

[0051] **FIG. 10** illustrates a transmembrane hidden Markov model prediction for the hD5D gene;

[0052] **FIG. 11** shows the multiple alignments for fatty acid desaturases of different organisms highlighting the cytochrome b_5 motif and conserved histidine boxes. Identical or highly conserved residues are shaded;

[0053] **FIG. 12** is a dendogram showing the similarities or relatedness of the three human fatty acid desaturases to fatty acid desaturases from other organisms;

[0054] **FIG. 13** is a schematic representation of plasmid pYr5003.1 (7104 bp). The rat delta-6-desaturase-1 coding sequence is shown between restriction sites for XbaI and HindIII;

[0055] **FIG. 14** is a schematic representation of plasmid pTr5404.1 (7207 bp) that contains the N-terminal tags. The rat delta-6-desaturase 1 coding sequence is shown between restriction sites for XbaI and HindIII;

[0056] **FIG. 15** is a schematic representation of plasmid pYh5001.2 (7116 bp). The human delta-6-desaturase 1 coding sequence is shown between restriction sites for XbaI and HindIII;

[0057] **FIG. 16** is a schematic representation of plasmid pTh5002.1 (7207 bp), which contains the N-terminal tags. The human delta-6-desaturase 1 coding sequence is shown between restriction sites for XbaI and HindIII;

[0058] **FIG. 17** is a schematic representation of plasmid pRr4001.1. The rat delta-6-desaturase 1 control region is shown between restriction sites for XhoI and SacI;

[0059] **FIG. 18** is a schematic representation of plasmid pRh4002.1. The human delta-6-desaturase 1 control region is shown between the two restriction sites for KpnI;

[0060] **FIG. 19** is a schematic representation of plasmid pGh4015.1 . The human delta-6-desaturase 1 control region is shown between restriction sites for XhoI and KpnI;

[0061] **FIG. 20** illustrates the expression of the CAT reporter gene under the control of the rat (pRr4001.1) or the human (pRh4002.1) delta-6-desaturase 1 gene control region as compared to its expression from the SV40 promoter (pCAT-3-CTL) after transfection in ZR-75-1 cells. The levels of expression were determined by the CAT enzymatic activity and expressed relative to the pCAT-3-CTL. The empty vector (pCAT-3-Enhancer) was also transfected as a negative control. Bars indicate standard deviation from three experiments;

[0062] **FIG. 21** shows a High Performance Liquid Chromatographic (HPLC) analysis of radiolabelled methyl esters of fatty acids from yeast transformed with pYES2 (panel A) or pYr5003.1 (panel B) incubated with linoleic acid, [1-^{14}C]-18:2n-6;

[0063] **FIG. 22** shows a gas chromatographic analysis of methyl esters of fatty acids from yeast transformed with pYES2 (panel A) or pYr5003.1 (panel B) incubated with linoleic acid, 18:2n-6. The arrow indicates the presence of a new fatty acid, gamma-linolenic acid, 18:3n-6. The common peaks to both yeast were identified as: a, 14:0; b, 16:0; c, 16:1n-7; d, 18:0; f, 18:1n-9;

[0064] **FIG. 23** shows the percent conversion of alpha-linolenic acid (18:3n-3) into 18:4n-3 in *Saccharomyces cerevisiae* cells transformed with pYr5003.1 at different induction time points with galactose;

[0065] **FIG. 24** illustrates the percentage of radioactivity from [1-^{14}C]-18:3n-3 recovered in spheroplasts and yeast whole cells transformed with pYr5003.1;

[0066] **FIG. 25** illustrates the percentage of radioactivity from [1-^{14}C]-24:4n-6 recovered in spheroplasts and yeast whole cells transformed with pYr5003.1; and

[0067] **FIG. 26** illustrates the percentage of delta-6-desaturation of [1-^{14}C]-18:3n-3 in spheroplasts and yeast whole cells transformed with pYr5003.1.

DETAILED DESCRIPTION OF THE INVENTION

[0068] The present invention has evolved from observations that oral supplementation of naturally occurring fatty acids has had some therapeutic benefit in counteracting existing metabolic deficiencies prevalent in certain disease conditions. Using this observation, nutritional and pharmaceutical products have hitherto been developed using oils rich in selected fatty acids.

[0069] However, to address new strategies for therapeutic intervention, it is necessary to go beyond the measurement of lipid levels and lipid supplementation and directly measure actual enzyme activities and the regulation of expression of the genes from which these enzymes are encoded. The human genes that are uniquely involved and responsible for expressing the various enzymes utilized along these pathways have hitherto been mostly uncharacterized.

[0070] In this regard, the development of an experimental model that can be manipulated to study the expression of genetic material isolated from humans and other species is beneficial in establishing the role and function which these genes and their encoded proteins exhibit in PUFA metabolism. This is particularly so in recognition of the fact that the relationship between a protein's unique role in a metabolic pathway and the expression of the gene encoding that protein is normally a well coordinated event such that subtle deviations can lead to abnormal physiological processes. Moreover, such a system would facilitate the discovery and identification of candidate drug targets effective in correcting abnormalities or imbalances in lipid metabolic changes associated with certain pathological conditions, such as diabetic neuropathy.

[0071] The invention teaches (1) the isolation, cloning and identification of the control region (i.e. promoter and other regulatory elements) of both a human and a rat fatty acid desaturase gene and (2) the use of the desaturase gene control region in drug screening methods to identify test components which can effectively modulate desaturase gene expression. The present invention incorporates the knowledge that the particular genetic elements, which are responsible for controlling desaturase gene expression, can be isolated independently of the desaturase gene encoding region (i.e. amino acid coding sequences) and, therefore, be employed to assay for agents that modulate desaturase gene expression.

[0072] The utility of such genetic control and regulatory elements ranges from their use as tissue specific promoters that drive gene expression to the fine-tuning of metabolic processes involved in biochemical pathways. Accordingly, cloning of the control regions of the desaturase genes provides a powerful tool for dissecting the role of desaturase gene expression and inducing modifications thereof, which can eliminate or control alterations associated with metabolic disorders. Therefore, the identification and characterization of the control regions of desaturase genes allow us to identify and understand the role of discrete regulatory elements located in desaturase control regions as well as to discover potential pharmacological modulators of desaturase gene expression.

[0073] Another object of the invention is to provide methods that are designed to screen for nucleotides, proteins, compounds or pharmacological agents that regulate the level of expression of the genes that encode fatty acid desaturase enzymes, i.e. various components that act as enhancers or inhibitors of desaturase gene expression and hence, modify the desaturase enzyme concentration in tissues. To this end, cell-based and/or cell lysate assays are used to detect components that modulate the transcriptional activity of the desaturase genes. Such experimental methods make it possible to screen large collections of natural or synthetic compounds for therapeutic agents that affect desaturase gene expression.

[0074] Therefore, an object of the present invention is to provide methods for the screening of nucleotides, proteins, compounds or pharmacological agents that modulate fatty acid desaturase enzyme activity, i.e. various components that act as enhancers or inhibitors of desaturation and hence, modify unsaturated fatty acid biosynthesis. To this end, cell-based and/or cell lysate assays are used to detect components that modulate the activity of the desaturase enzymes. Such experimental methods make it possible to screen large collections of natural or synthetic compounds for therapeutic agents that affect desaturase enzyme activity.

[0075] For example, when an unsaturated fatty acid metabolite is to be produced in vivo, the substrate for the corresponding fatty acid desaturase will normally already be

present. In the case where the enzymatic activity of a native fatty acid desaturase is altered, the administration of an appropriate therapeutic agent can remedy this alteration through its direct action on the enzyme. As a result, the native desaturase can ultimately act on its substrate, already present in the cell, and in vivo synthesis of the required fatty acid product is achieved. Accordingly, desaturase activity can either be restored or increased in conditions where such activity essential to fatty acid biosynthesis is abnormal.

[0076] Similarly, in the case where expression of a native desaturase gene is reduced, the administration of an appropriate therapeutic agent can remedy this effect through its direct action on functional or regulatory elements within the control region of the desaturase gene. As a result, increased expression of the gene takes place and hence, in vivo synthesis of the required desaturase enzyme is restored or increased in conditions where such activity essential to fatty acid biosynthesis is abnormal.

[0077] Therefore, isolated nucleic acid sequences encoding these desaturase enzymes have utility in constructing in vivo and/or in vitro experimental models for identifying test components which modulate mammalian fatty acid desaturase activity and/or the level and regulation of desaturase gene expression. Furthermore, the modulation or regulation of fatty acid desaturase enzyme activity or gene expression by various test components will be identified by the methods disclosed herein and hence, be used to reduce disease processes or symptoms.

[0078] It is understood that the present invention is not limited to the particular methodology, protocols, cell lines, vectors, and reagents described herein. Generally, the laboratory procedures in cell culture and molecular genetics described below are those well known and commonly employed in the art. Standard techniques are used for recombinant nucleic acid methods, polynucleotide synthesis, microbial culture, transformation, transfection, etc. Generally, enzymatic reactions and purification steps are performed according to the manufacturer's specifications. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods, devices, and materials are described below.

[0079] Definitions

[0080] The following illustrative explanations are provided to facilitate understanding of certain terms used frequently herein. The explanations are provided as a convenience and are not limitative of the invention.

[0081] Agonist refers to any molecule or pharmaceutical agent, such as a drug or hormone, which enhances the activity of another molecule.

[0082] Antagonist refers to any molecule or pharmaceutical agent, such as a drug or hormone, which inhibits or extinguishes the activity of another molecule.

[0083] Chemical Derivative. As used herein, a molecule is the to be a "chemical derivative" of another molecule when it contains additional chemical moieties not normally a part of the molecule. Such moieties can improve the molecule's solubility, absorption, biological half-life, and the like. The moieties can alternatively decrease the toxicity of the molecule, eliminate or attenuate any undesirable side effect of the molecule, and the like. Moieties capable of mediating such effects are disclosed in Mack E. W., 1990, *Remington's Pharmaceutical Sciences*, Mack Publishing Company, Easton, Pa., 13th edition. Procedures for coupling such moieties to a molecule are well known in the art.

[0084] C mpositions include genes, proteins, polynucleotides, peptides, compounds, drugs, and pharmacological agents.

[0085] Control region refers to a nucleic acid sequence capable of; or required for, assisting or impeding initiation, termination, or otherwise regulating the transcription of a gene. The control region may include a promoter, enhancer, silencer and/or any other regulatory element A control region also includes a nucleic acid sequence that may or may not be independently or exclusively sufficient to initiate, terminate, or otherwise regulate transcription, however, is capable of effecting such regulation in association with other nucleic acid sequences.

[0086] Delta-5-Desaturase (D5D) is an enzyme that is capable of introducing a double bond between carbons 5 and 6 from the carboxyl group in a fatty acid molecule.

[0087] Delta-6-Desaturase (D6D) is an enzyme which is capable of introducing a double bond between carbons 6 and 7 from the carboxyl group in a fatty acid molecule.

[0088] Desaturase refers to a fatty acid desaturase, which is an enzyme capable of generating a double bond in the hydrocarbon region of a fatty acid molecule.

[0089] Disorder as used herein refers to derangement or abnormality of structure or function. Disorder includes disease.

[0090] Drug. Drugs include, but are not limited to proteins, peptides, degenerate peptides, agents purified from conditioned cell medium, organic molecules, inorganic molecules, antibodies or oligonucleotides. The drug can be naturally occurring or synthetically or recombinantly produced.

[0091] Enhancer is a nucleic acid sequence comprising a DNA regulatory element that enhances or increases transcription when bound by a specific transcription factor or factors. Moreover, an enhancer may function in either orientation and in any location (upstream or downstream relative to the promoter) to effect and generate increased levels of gene expression when bound by specific factors. In addition, according to the present invention, an enhancer also refers to a compound (i.e. test compound) that increases or promotes the enzymatic activity of the fatty acid regulated gene, and/or increases or promotes the transcription of the gene.

[0092] Fatty Acids are a class of compounds comprising a long saturated or mono or polyunsaturated hydrocarbon chain and a terminal carboxyl group.

[0093] Functional Derivative. A "functional derivative" of a sequence, either protein or nucleic acid, is a molecule that possesses a biological activity (either functional or structural) that is substantially similar to a biological activity of the protein or nucleic acid sequence. A functional derivative of a protein can contain post-translational modifications such as covalently linked carbohydrate, depending on the necessity of such modifications for the performance of a

specific function. The term "functional derivative" is intended to include the "fragments,""segments,""variants, ""analogs," or "chemical derivatives" of a molecule.

[0094] Gene refers to a nucleic acid molecule or a portion thereof, the sequence of which includes information required for the production of a particular protein or polypeptide chain. A full-length sequence or any portion of the coding sequence can encode the polypeptide, so long as the functional activity of the protein is retained. A gene may comprise regions preceding and following the coding region as well as intervening sequences (introns) between individual coding segments (exons). A "heterologous" region of a nucleic acid construct (i.e. a heterologous gene) is an identifiable segment of DNA within a larger nucleic acid construct that is not found in association with the other genetic components of the construct in nature. Thus, when the heterologous gene encodes a mammalian fatty acid regulated gene, a promoter that does not flank the structural genomic DNA in the genome of the source organism will usually flank the gene.

[0095] Host system may comprise a cell, tissue, organ, organism or any part thereof, which provides an environment or conditions that allow for, or enable, transcription and/or transcription.

[0096] Identity, similarity, homology or homologous, refer to relationships between two or more polynucleotide sequences, as determined by comparing the sequences. In the art, identity also means the degree of sequence relatedness between polynucleotide sequences, as the case may be, as determined by the match between strings of such sequences. Both identity and similarity can be readily calculated (Lesk A.M., ed., 1988, *Computational Molecular Biology*, Oxford University Press, NY; Smith D. W., ed., 1993, *Biocomputing: Informatics and Genome Project*, Academic Press, NY; Griffin A. M. and Griffin H. G., eds., 1994, *Computer Analysis of Sequence Data, Part* 1, Humana Press, NJ; von Heijne G., 1987, *Sequence Analysis in Molecular Biology*, Academic Press, NY and Gribskov M. and Devereux J., eds., 1991, *Sequence Analysis Primer*, M Stockton Press, NY). While there exist a number of methods to measure identity and similarity between two polynucleotide sequences, both terms are well known to skilled artisans (von Heijne G., 1987, *Sequence Analysis in Molecular Biology*, Academic Press, NY; Gribskov M. and Devereux J., eds., 1991, *Sequence Analysis Primer*, M Stockton Press, NY; Carillo H. and Lipman D., 1988, *SIAM J. Applied Math.*, 48: 1073). Methods commonly employed to determine identity or similarity between sequences include, but are not limited to those disclosed in Carillo H. and Lipman D., 1988, *SIAM J. Applied Math.*, 48: 1073. Methods to determine identity and similarity are codified in computer programs. Computer program methods to determine identity and similarity between two sequences include, but are not limited to, GCG program package (Devereux et al., 1984, *Nucl. Acid Res.*, 12: 387-395), BLASTP, BLASTN and FASTA (Altschul et al., 1990, *J. Molec. Biol*, 215: 403-410).

[0097] Isolated means altered "by the hand of man" from its natural state; i.e., that, if it occurs in nature, it has been changed or removed from its original environment, or both. For example, a naturally occurring polynucleotide naturally present in a living organism in its natural state is not "isolated," but the same polynucleotide separated from coexisting materials of its natural state is "isolated", as the term is employed herein. As part of or following isolation, such polynucleotides can be joined to other polynucleotides, such as DNA, for mutagenesis, to form fusion proteins, and for propagation or expression in a host, for instance. The isolated polynucleotides, alone or joined to other polynucleotides such as vectors, can be introduced into host cells, in culture or in whole organisms. Introduced into host cells in culture or in whole organisms, such DNA still would be isolated, as the term is used herein, because they would not be in their naturally occurring form or environment. Similarly, the polynucleotides may occur in a composition, such as a media formulations, solutions for introduction of polynucleotides, for example, into cells, compositions or solutions for chemical or enzymatic reactions, for instance, which are not naturally occurring compositions, and, therein remain isolated polynucleotides within the meaning of that term as it is employed herein.

[0098] Mutation. A "mutation" is any detectable change in the genetic material. A mutation can be any (or a combination of) detectable, unnatural change affecting the chemical or physical constitution, mutability, replication, phenotypic function, or recombination of one or more deoxyribonucleotides; nucleotides can be added, deleted, substituted for, inverted, or transposed to new positions with and without inversion. Mutations can occur spontaneously and can be induced experimentally by application of mutagens or by site directed mutagenesis. A mutant polypeptide can result from a mutant nucleic acid molecule.

[0099] Nucleic acid construct refers to any genetic element, including, but not limited to, plasmids and vectors, that incorporate polynucleotide sequences. For example, a nucleic acid construct may be a vector comprising a promoter or control region that is operably linked to a heterologous gene.

[0100] Operably linked as used herein indicates the association of a promoter or control region of a nucleic acid construct with a heterologous gene such that the presence or modulation of the promoter or control region influences the transcription of the heterologous gene, including genes for reporter sequences. Operably linked sequences may also include two segments that are transcribed onto the same RNA transcript. Thus, two sequences, such as a promoter and a "reporter sequence" are operably linked if transcription commencing in the promoter produces an RNA transcript of the reporter sequence.

[0101] Plasmids. Starting plasmids disclosed herein are either commercially available, publicly available, or can be constructed from available plasmids by routine application of well-known, published procedures. Many plasmids and other cloning and expression vectors that can be used in accordance with the present invention are well known and readily available to those of skill in the art. Moreover, those of skill readily may construct any number of other plasmids suitable for use in the invention.

[0102] Polynucleotides(s) of the present invention may be in the form of RNA, such as mRNA, or in the form of DNA, including, for instance, cDNA and genomic DNA obtained by cloning or produced by chemical synthetic techniques or by a combination thereof. The DNA may be double-stranded or single-stranded. Single-stranded polynucleotides may be

the coding strand, also known as the sense strand, or it may be the non-coding strand, also referred to as the anti-sense strand. Polynucleotides generally refer to any polyribonucleotide or polydeoxribonucleotide, which may be unmodified RNA or DNA or modified RNA or DNA. Thus, for instance, polynucleotides as used herein refers to, among others, single-and double-stranded DNA, DNA that is a mixture of single- and double-stranded regions or single-, double- and triple-stranded regions, single- and double-stranded RNA, and RNA that is mixture of single- and double-stranded regions, hybrid molecules comprising DNA and RNA that may be single-stranded or, more typically, double-stranded, or triple-stranded, or a mixture of single- and double-stranded regions. In addition, polynucleotide as used herein refers to triple-stranded regions comprising RNA or DNA or both RNA and DNA. The strands in such regions may be from the same molecule or from different molecules. The regions may include all of one or more of the molecules, but more typically involve only a region of some of the molecules. One of the molecules of a triple-helical region often is an oligonucleotide. As used herein, the term polynucleotide also includes DNA or DNA that contain one or more modified bases. Thus, DNA or DNA with backbones modified for stability or for other reasons are "polynucleotides" as that term is intended herein. Moreover, DNA or DNA comprising unusual bases, such as inosine, or modified bases, such as tritylated bases, to name just two examples, are polynucleotides as the term is used herein. It will be appreciated that a great variety of modifications have been made to DNA and RNA that serve many useful purposes known to those of skill in the art. The term polynucleotide as it is employed herein embraces such chemically, enzymatically or metabolically modified forms of polynucleotides, as well as the chemical forms of DNA and RNA characteristic of viruses and cells, including simple and complex cells, inter alia. Polynucleotides embraces short polynucleotides often referred to as oligonucleotide(s). It will also be appreciated that RNA made by transcription of this doubled stranded nucleotide sequence, and an antisense strand of a nucleic acid molecule of the invention or an oligonucleotide fragment of the nucleic acid molecule, are contemplated within the scope of the invention. An antisense sequence is constructed by inverting the sequence of a nucleic acid molecule of the invention, relative to its normal presentation for transcription. Preferably, an antisense sequence is constructed by inverting a region preceding the initiation codon or an unconserved region. The antisense sequences may be constructed using chemical synthesis and enzymatic ligation reactions using procedures known in the art.

[0103] Promoter refers to a nucleic acid sequence comprising a DNA regulatory element capable of binding RNA polymerase directly or indirectly to initiate transcription of a downstream (3' direction) gene. In accordance with the present invention, a promoter of a nucleic acid construct that includes a nucleotide sequence, wherein the nucleotide sequence may be linked to a heterologous gene such that the induction of the promoter influences the transcription of the heterologous gene.

[0104] Purified. A "purified" protein or nucleic acid is a protein or nucleic acid preparation that is generally free of contaminants, whether produced recombinantly, chemically synthesized or purified from a natural source.

[0105] Recombinant refers to recombined or new combinations of nucleic acid sequences, genes, or fragments thereof which are produced by recombinant DNA techniques and are distinct from a naturally occurring nucleic acid sequence

[0106] Regulatory element refers to a deoxyribonucleotide sequence comprising the whole, or a portion of, a nucleic acid sequence to which an activated transcriptional regulatory protein, or a complex comprising one or more activated transcriptional regulatory proteins, binds so as to transcriptionally modulate the expression of an associated gene or genes, including heterologous genes.

[0107] Reporter gene is a nucleic acid coding sequence whose product is a polypeptide or protein that, is not otherwise produced by the host cell or host system, or which is produced in minimal or negligible amounts in the host cell or host system, and which is detectable by various known methods such that the reporter gene product may be quantitatively assayed to analyze the level of transcriptional activity in a host cell or host system. Examples include genes for luciferase, chloramphenicol acetyl transferase (CAT), beta-galactosidase, secreted placental alkaline phosphatase and other secreted enzymes.

[0108] Silencer refers to a nucleic acid sequence or segment of a DNA control region such that the presence of the silencer sequence in the region of a target gene suppresses the transcription of the target gene at the promoter through its actions as a discrete DNA segment or through the actions of trans-acting factors that bind to these genetic elements and consequently effect a negative control on the expression of a target gene.

[0109] Stringent hybridization conditions are those which are stringent enough to provide specificity, reduce the number of mismatches and yet are sufficiently flexible to allow formation of stable hybrids at an acceptable rate. Such conditions are known to those skilled in the art and are described, for example, in Sambrook et al., 1989, *Molecular Cloning, 2nd Edition*, Cold Spring Harbor Laboratory Press, Cold Spring Harbour, NY or Ausubel et al., 1994-, *Current Protocols in Molecular Biology*, John Wiley & Sons, NY. By way of example only, stringent hybridization with short nucleotides may be carried out at 5-10° C. below the T_M using high concentrations of probe such as 0.01-1.0 pmole/ml. Preferably, the term "stringent conditions" means hybridization will occur only if there is at least 95% and preferably at least 97% identity between the sequences.

[0110] Tag refers to a specific short amino acid sequence, or the oligonucleotide sequence that encodes it, wherein the amino acid or nucleic acid sequence may comprise or encode, for example, a c-myc epitope and/or a string of six histidine residues recognizable by commercially available antibodies. In practice, a tag facilitates the subsequent identification and purification of a tagged protein.

[0111] Tagged protein as used herein refers to a protein comprising a linked tag sequence. For example, a tagged protein includes a mammalian fatty acid regulated polypeptide linked to a c-myc epitope and six histidine residues at the carboxyl terminus of the amino acid sequence.

[0112] Test compounds as used herein encompass small molecules (e.g. small organic molecules), pharmacological

compounds or agents, peptides, proteins, antibodies or antibody fragments, and nucleic acid sequences, including DNA and RNA sequences.

[0113] Transfection refers to a process whereby exogenous or heterologous DNA (i.e. a nucleic acid construct) is introduced into a recipient eukaryotic host cell. Therefore, in eukaryotic cells, the acquisition of exogenous DNA into a host cell is referred to as transfection. In prokaryotes and eukaryotes (for example, yeast and mammalian cells) introduced DNA may be maintained on an episomal element such as a plasmid or integrated into the host genome. With respect to eukaryotic cells, a stably transfected cell is one in which the introduced DNA has become integrated into a chromosome so that it is inherited by daughter cells through chromosome replication. This stability is demonstrated by the ability of the eukaryotic cell to establish cell lines or clones comprised of a population of daughter cells containing the introduced DNA.

[0114] Transformation refers to a process whereby exogenous or heterologous DNA (i.e. a nucleic acid construct) is introduced into a recipient prokaryotic host cell. Therefore, in prokaryotic cells, the acquisition of exogenous DNA into a host cell is referred to as transformation. Transformation in eukaryotes refers to the conversion or transformation of eukaryotic cells to a state of unrestrained growth in culture, resembling a tumorigenic condition. In prokaryotes and eukaryotes (for example, yeast and mammalian cells) introduced DNA may be maintained on an episomal element such as a plasmid or integrated into the host genome. With prokaryotic cells, a stably transformed bacterial cell is one in which the introduced DNA has become integrated into a chromosome so that it is inherited by daughter cells through chromosome replication. This stability is demonstrated by the ability of the prokaryotic cell to establish cell lines or clones comprised of a population of daughter cells containing the introduced DNA.

[0115] Transfection/transformation as used herein refers to a process whereby exogenous or heterologous DNA (e.g. a nucleic acid construct) has been introduced into a eukaryotic or prokaryotic host cell or into a host system.

[0116] Variant(s) of polynucleotides are polynucleotides that differ in nucleotide sequence from another, reference polynucleotide. A "variant" of a protein or nucleic acid is meant to refer to a molecule substantially similar in structure and biological activity to either the protein or nucleic acid. Thus, provided that two molecules possess a common activity and can substitute for each other, they are considered variants as that term is used herein even if the composition or secondary, tertiary, or quaternary structure of one of the molecules is not identical to that found in the other, or if the amino acid or nucleotide sequence is not identical. Generally, differences are limited so that the nucleotide sequences of the reference and the variant are closely similar overall and, in many regions, identical. Changes in the nucleotide sequence of the variant may be silent. That is, they may not alter the amino acids encoded by the polynucleotide. Where alterations are limited to silent changes of this type a variant will encode a polypeptide or polynucleotide with the same amino acid sequence as the reference. Changes in the nucleotide sequence of the variant may alter the amino acid sequence of a polypeptide encoded by the reference polynucleotide. Such nucleotide changes may result in amino acid substitutions, additions, deletions, fusions and truncations in the polypeptide or polynucleotide encoded by the reference sequence.

[0117] Vector is a plasmid or phage DNA or other DNA sequence into which DNA can be inserted to be cloned. The vector can replicate autonomously in a host cell, and can be further characterized by one or a small number of endonuclease recognition sites at which such DNA sequences can be cut in a determinable fashion and into which DNA can be inserted. The vector can further contain a marker suitable for use in the identification of cells transformed with the vector. Markers, for example, are tetracycline resistance or ampicillin resistance. The words "cloning vehicle" are sometimes used for "vector."

[0118] It will be appreciated that the invention includes nucleotide or amino acid sequences which have substantial sequence homology with, respectively, the subject polynucleotides or polypeptides. The term "sequences having substantial sequence homology" means those nucleotide and amino acid sequences which have slight or inconsequential sequence variations from the subject polynucleotides; i.e. the homologous sequences function in substantially the same manner to produce substantially the same polypeptides as the actual sequences. The variations may be attributable to local mutations or structural modifications. It is expected that a sequence having 85-90% sequence homology with the DNA sequence of the invention will provide functional subject polypeptides which retain substantially the same biological function or activity as the polynucleotide encoded by the subject polynucleotides. Further embodiments of the invention are polynucleotides that are at least 70% identical over their entire length to a subject polynucleotide, and polynucleotides which are complementary to such polynucleotides. Other embodiments are polynucleotides that comprise a region that is at least 80% identical over their entire length to a subject polynucleotide and polynucleotides complementary thereto. This includes polynucleotides at least 90% identical over their entire length to the same, and among these embodiments are polynucleotides with at least 95% homology. Furthermore, those with at least 97% are highly preferred among those with at least 95%, and among these those with at least 98% and at least 99% are particularly highly preferred, with at least 99% being the more preferred.

[0119] The present invention encompasses the use of individual coding (e.g. open reading frame) and non-coding portions (e.g. control region) of mammalian desaturase genes, preferably human and rat desaturase genes, in recombinant DNA constructs to enable their expression/operability in host systems for drug screening purposes.

[0120] In accordance with the present invention, nucleic acid sequences which encode fatty acid desaturases, fragments of the nucleic acid sequences, tagged protein sequences or functional equivalents thereof may be used in recombinant DNA constructs that direct the expression of desaturases in appropriate host systems. Due to the inherent degeneracy of the genetic code, other DNA sequences, which encode substantially the same or a functionally equivalent amino acid sequence, may be used to clone and express mammalian fatty acid desaturases.

[0121] Furthermore, the nucleic acid sequences of the present invention can be engineered in order to alter a

desaturase coding or control sequence for a variety of reasons, including but not limited to, alterations which modify the cloning, processing and/or expression of the gene product. For example, mutations may be introduced using techniques, which are well known in the art, e.g. site-directed mutagenesis to introduce endonuclease recognition sites, to alter glycosylation patterns, to change codon preference, etc.

[0122] In a particular embodiment, the invention encompasses polynucleotides encoding a functional rat delta-6-desaturase (rD6D-1) and a human delta-6-desaturase (hD6D-1) having the nucleic acid sequences illustrated in **FIGS. 3 and 4**, respectively. The deduced amino acid sequences encoded by the nucleic acid sequences of rD6D-1 and hD6D-1 are illustrated in **FIG. 5** and **FIG. 6** respectively.

[0123] In another embodiment, the invention encompasses novel oligonucleotides comprising the control region of rD6D-1 and hD6D-1 which are represented by the nucleic acid sequences of **FIGS. 1 and 2**, respectively (SEQ ID NOS:1 and 2).

[0124] In yet another embodiment of the invention, a nucleic acid sequence encoding a mammalian desaturase is ligated to a heterologous sequence (e.g. tag or tags) to encode a tagged desaturase. A tagged desaturase is easily identified through the use of an antibody, which will specifically recognize and bind to the heterologous portion of the tagged fatty acid desaturase. Accordingly, a tagged desaturase is beneficial in determining whether the mammalian desaturase has been appropriately expressed in a host system. The carboxyl terminal end of the mammalian desaturase polypeptide is ligated to a stretch of amino acid residues containing tags and in the present invention, is preferably the V5 and the 6×His epitope tags which have the amino acid sequences represented as GKPIPNPLLGLDST and HHHHHH-COOH, respectively. The single-letter code for amino acids used is as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S. Ser; T, Thr, V, Val; W, Trp; Y, Tyr.

[0125] Furthermore, a tagged fatty acid desaturase may be engineered to contain a cleavage site located between the desaturase amino acid sequence and the heterologous sequence (e.g. the tag), so that the desaturase may be cleaved away from the heterologous moiety after purification. For example, a system described by Janknecht et al allows for the ready purification of non-denatured tagged proteins expressed in human cell lines (Janknecht et al., 1991, *Proc. Natl. Acad Sci*, USA, 88: 8972-8976). In this system, the gene of interest is subcloned into a vaccinia recombination plasmid such that the gene's open reading frame is translationally fused to an amino-terminal tag consisting of six histidine residues (6×His). Extracts from cells infected with recombinant vaccinia virus are loaded onto Ni^{2+} nitrilotriacetic acid-agarose columns and histidine-tagged proteins are selectively eluted with imidazole-containing buffers.

[0126] Cloning of Mammalian Desaturase Genes and Control Regions

[0127] Techniques for cloning, sequencing, expressing and purifying polypeptides are well known to the skilled person. Various techniques are disclosed in standard textbooks, such as Sambrook et al., 1989, *Molecular Cloning, A Laboratory Manual, 2nd edition*, Cold Spring Harbour Press, Cold Spring Harbour, N.Y.; Old R. W. and Primrose S. B., 1994, *Principles of Gene Manipulation, 5th Edition*, Blackwell Scientific Publications, U.S.A.; and Ausubel et al., 1994-, *Current Protocols in Molecular Biology*, John Wiley & Sons, New York, N.Y.

[0128] Primers may be designed using the Primer Premier software (Premier Biosoft International, Palo Alto, Calif.), Vector NTI (Informax, Inc., North Bethesda, Md.), OLIGO 4.06 Primer Analysis software (National Biosciences Inc., Plymouth, Minn.), or another appropriate program. Alternatively, the selected primer can be chosen based on cloning strategy without the aid of any software.

[0129] Methods for DNA sequencing are well known in the art and employ such enzymes or commercially available kits as SEQUENASE (US Biochemical Corp, Cleveland Ohio)), Taq polymerase (Perkin Elmer, Norwalk Conn.), thermostable T7 polymerase (Amersham, Chicago Ill.), or combinations of recombinant polymerases and proofreading exonucleases such as the ELONGASE amplification system marketed by Gibco BRL (Gaithersburg Md.). Preferably, the process is automated with instruments such as the LiCor DNA Sequencer Long Readir 4200, the Hamilton MICROLAB 2200 (Hamilton, Reno Nev.), Peltier thermal cycler (PTC200; MJ Research, Watertown Me.) or the ABI 377 DNA sequencers (Perkin Elmer).

[0130] Mammalian Desaturase Genes

[0131] According to the present invention, cDNAs were prepared from mRNA using RT-PCR (reverse transcriptase-polymerase chain reaction) (PCR Protocols: A Guide to Methods and Applications, Innis, M., et al., Academic Press (1990), San Diego, Calif.) employing oligonucleotide forward and reverse primers. Initially, cDNA was generated through reverse transcription of total RNA that was extracted from tissue specific for expressing mammalian fatty acid desaturases using a set of random primers (Perkin-Elmer). Subsequent amplification of desaturase cDNA was achieved by PCR using forward and reverse primers specifically designed to correspond to the coding sequences for the rD6D-1 and hD6D-1 genes, i.e. a forward primer which will hybridize or bind to the 5'-translated region of the antisense strand of the rD6D-1 or hD6D-1 encoding cDNA and a reverse primer which will hybridize or bind to the 3'-translated region of the sense strand of the same desaturase cDNA molecule.

[0132] The oligonucleotide primers designed for amplification of mammalian desaturase cDNA may advantageously comprise one or more endonuclease recognition sites to facilitate cloning into an expression vector following amplification by PCP In the present invention, the forward and reverse primers used for cloning the mammalian desaturase genes contain a HindIII and a XbaI restriction site, respectively.

[0133] Optionally, an oligonucleotide primer may lack a translation initiation or termination codon so long as such codons are provided in the cloning vector, which need be operatively associated with the cDNA sequence encoding the mammalian desaturase (i.e. positioned upstream at the 5'-end or downstream at the 3'-end of the desaturase encoding sequence, respectively). In a preferred embodiment of

the present invention, the translation initiation and termination codons are provided within the forward and reverse primer sequences, respectively, the exception being that the primers used to create the tagged constructs lacked termination codons.

[0134] Examples of forward and reverse primers that are useful in cloning rD6D-1 and hD6D-1 cDNAs for insertion into expression vectors are listed below in Table 1. The endonuclease recognition sites are underlined and the translation initiation and termination codons are indicated in boldface type.

TABLE 1

Forward - rD6D-1	5'-CACGCGAAGCTTATGGGGAAGGGAGGTAACCAG-3'
Reverse - rD6D-1	5'-CACGCGTCTAGATCATTTGTGGAGGTAGGCATC-3'
Reverse - rD6D-1	5'-CACGCGTCTAGATTTGTGGAGGTAGGCATCCAG-3'
Forward - hD6D-1	5'-CACGCGAAGCTTATGGGGAAGGGAGGGAAC-3'
Reverse - hD6D-1	5'-CACGACTCTAGAGGGGCTGTGGCTTCATTTGT-3'
Reverse - hD6D-1	5'-CACGCGTCTAGATTTGTGAAGGTAGGCGTCCAG-3'

[0135] In a preferred embodiment of the invention, an rD6D-1 cDNA fragment (1.3 kb) spanning nucleotides +1 to +1335 was cloned by reverse transcription and PCR-amplification of total RNA extracted from rat liver tissue. To this end, the nucleotide sequence that encodes a functionally active rD6D-1 is depicted in **FIG. 3**. The encoded rD6D-1 is represented by the amino acid sequence depicted in **FIG. 5**.

[0136] In another preferred embodiment of the invention, an hD6D-1 cDNA fragment (1.3 kb) spanning nucleotides +1 to +1335 was cloned from the human cell line Chang (ATCC No.CCl-13) by reverse transcription and PCR-amplification of total RNA. To this end, the nucleotide sequence that encodes a functionally active hD6D-1 is depicted in **FIG. 4**. The encoded hD6D-1 is represented by the amino acid sequence depicted in **FIG. 6**.

[0137] Mammalian Desaturase Vector Constructs

[0138] Methods which are well known to those skilled in the art may be used to construct expression vectors containing nucleic acid sequences encoding mammalian desaturases and appropriate transcriptional and translational control elements. These methods include in vitro recombinant DNA techniques, synthetic techniques, and in vivo genetic recombination. Such techniques are described in Sambrook et al., 1989, *Molecular Cloning, A Laboratory Manual,* 2nd *edition*, Cold Spring Harbour Press, Cold Spring Harbour, N.Y. and Ausubel et al., 1994-, *Current Protocols in Molecular Biology*, John Wiley & Sons, New York, N.Y

[0139] It will be understood that not all vectors, expression control sequences and hosts will function equally well to express the DNA sequences of this invention. Neither will all hosts function equally well with the same expression system. However, one skilled in the art will be able to select the proper vectors, expression control sequences, and hosts without undue experimentation to accomplish the desired expression without departing from the scope of this invention. For example, in selecting a vector and/or an expression control sequence, a variety of factors will normally be considered. These include, for example, the relative strength of the expression, its controllability, and its compatibility with the particular nucleic acid sequence or gene to be expressed. Furthermore, in selecting a vector, the host must also be considered because the vector must be maintained and be functional within it. The vector's copy number, the ability to control that copy number, and the expression of any other proteins encoded by the vector, such as antibiotic markers, will also be considered.

[0140] The expression control sequence is the non-translated region of the vector (eg. enhancers, promoters, and 5' and 3' untranslated regions) which interact with host cellular proteins to carry out transcription and translation. Such elements may vary in their strength and specificity. Depending on the vector system and host utilized, any number of suitable transcription and translation elements, including constitutive and inducible promoters, may be used. For example, when cloning in bacterial systems, inducible promoters such as the T7 promoter of pET9 (Promega), temperature sensitive promoters, or an osmotically sensitive promoter of pOSEX (Herbst et al., 1994, *Gene,* 151: 137-142) and the like may be used. The baculovirus polyhedrin promoter and the like may be used in insect cells. Promoters or enhancers derived from the genomes of plant cells (e.g. heat shock, RUBISCO, and storage protein genes) or from plant viruses (e.g., viral promoters or leader sequences) may be cloned into the vector. In mammalian cell systems, promoters from mammalian genes or mammalian viruses are preferable.

[0141] Suitable hosts will be selected by consideration of their compatibility with the chosen vector, their secretion characteristics, their ability to fold proteins correctly, the toxicity to the host of the product encoded by the DNA sequences to be expressed, and the ease of purification of the expression products.

[0142] Considering these and other factors a person skilled in the art will be able to construct a variety of vector/expression control sequence/host combinations that may be utilized to contain and express nucleic acid sequences encoding mammalian desaturases. Examples of hosts include, but are not limited to, micro-organisms such as bacteria or yeast, insect cell systems; plant cell systems or animal cell systems tansformed/transfected with appropriate expression vectors. A person skilled in the art will be able to introduce the constructs into the appropriate host and propagate the host.

[0143] Expression Vectors for Rat and Human Desaturase Genes

[0144] In order to express a functionally active mammalian desaturase, the nucleic acid sequence encoding the desaturase is inserted into an appropriate expression vector, i.e. a vector which contains the necessary elements for the transcription and translation of the inserted coding sequence. Depending on the vector system and host utilized, any number of suitable transcription and translation elements may be used.

[0145] A range of host systems may be utilized to harbour and express nucleic acid sequences encoding mammalian desaturases. Examples of hosts may include well known prokaryotic hosts, such as strains of *E. coli, Pseudomonas putida* and *Bacillus subtilis*; fungi such as yeasts (*Saccharomyces cerevisiae*, and methylotrophic yeast such as *Pichia pastoris, Hansenula polymorpha, Kluyveromyces lactis* and *Schizosaccharomyces pombe*); mammalian cells, such as CHO, African Green Monkey kidney cells (e.g., COS 1, BSC1, BSC40, and BMT10); insect cells (e.g., Sf9); and human cells in tissue culture.

[0146] In a preferred embodiment, *E. coli* is the specific prokaryotic host for cloning and replicating the DNA sequence of the present invention. On the other hand, yeast, in particular *Saccharomyces cerevisiae*, is the preferred host used for expression of mammalian desaturase coding sequences.

[0147] Accordingly, a vector construct of the present invention includes essential elements for its proliferation and selection in both eukaryotic and prokaryotic cells. Preferred expression vectors of the invention are pYES2 and pYES2/CT (Invitrogen) which essentially comprise an origin of replication, an inducible promoter and two selectable marker genes. In particular, the pYES2/CT vector also contains a short DNA sequence that encodes for tags (e.g. V5/6×HIS epitopes) which allow the translated product, a tagged desaturase protein, to be easily identified and/or purified using commercially available antibodies and/or affinity chromatography columns. The pYES2 and pYES2/CT vectors, confer uracil prototrophy for selection in yeast, and a GAL1 galactose-inducible promoter for expression which is activated in the presence of galactose and situated upstream of the cloning site. Galactose-inducible promoters (GAL1, GAL7, and GAL10) have been extensively utilized for high level and regulated expression of proteins in yeast (Lue et al., 1987, *Mol. Cell. Biol.*, 7: 3446-3451 and Johnston M., 1987, *Microbiol Rev.*, 51: 458-476). Transcription from the GAL promoters is activated by the GAL4 protein, which binds to the promoter region and activates transcription when galactose is present. In the absence of galactose, the antagonist GAL80 binds to GAL4 and prevents GAL4 from activating transcription. Addition of galactose prevents GAL80 from inhibiting activation by GAL4.

[0148] While it is not essential, optionally an expression vector may comprise a translation initiation or termination (e.g. stop) sequence oriented and operatively associated with the cDNA sequence encoding the mammalian desaturase (i.e. positioned upstream at the 5'-end or downstream at the 3'-end of the desaturase coding sequence, respectively). In a preferred embodiment, the translation initiation and termination codons are already provided within the forward and reverse primer sequences, respectively, which are used to facilitate cloning of the mammalian desaturase genes into the pYES2 vector (see Table 8). Forward and reverse primers for cloning into pYES2/CT are designed to express a desaturase-V5/6×His tagged protein (see Table 8).

[0149] The transformed/transfected host cell can be identified by selection for a marker gene contained on the introduced vector construct. The introduced marker gene, therefore, may confer antibiotic resistance, or encode an essential growth factor or enzyme, and permit growth on selective media when expressed in the transformed/transfected host. Typically, transformed/transfected hosts are selected due to their ability to grow on selective media. Selective media may contain an antibiotic or lack an essential growth nutrient necessary for the growth of the untransformed/untransfected host According to the invention, transformation of *E. coli* cells and yeast cells was determined through selection on ampicillin-containing medium and uracil-deficient medium, respectively, based on the selection marker genes (e.g. beta-lactamase and URA3) present in the pYES2 and pYES2/CT vectors.

[0150] A microsomal host system may be achieved by transforming/transfecting the host system with the nucleic acid construct containing the coding sequence for a functional mammalian desaturase described above, and isolating microsomes. Microsomal systems have been used successfully for testing enzyme activity from a number of different sources such as animal organs including liver, brain, heart, etc. and micro-organisms including yeast (de Antueno et al., 1994, *Lipids*, 29: 327-331, Todd et al., 1999, *Plant J.*, 17: 119-130, and Nishi et al., 2000, *Biochim. Biophys. Acta.*, 1490: 106-108).

[0151] Alternatively, an in vitro expression system can be accomplished, for example, by placing the nucleic acid sequence of the coding region for a functional mammalian desaturase polypeptide, described above, in an appropriate expression vector designed for in vitro use. In vitro transcription/translation can be carried out by adding rabbit reticulocyte lysate and essential cofactors; labelled amino acids can be incorporated if desired (Promega Corp., WI). Such in vitro expression vectors may provide some or all of the expression signals necessary in the system used. These methods are well known in the art and the components of the system are commercially available. The reaction mixture can then be assayed directly for the polypeptide, for example by determining its specific enzymatic activity, or the synthesized polypeptide can be purified and then assayed for its specific enzymatic activity.

[0152] Reporter Vectors for Rat and Hunan Control Regions

[0153] In order to identify the discrete control elements involved in the regulation of mammalian desaturase gene expression, a vector construct comprising a heterologous nucleic acid sequence encoding a reporter gene operably linked to a desaturase control region is used which is compatible to and sufficient for use in a host system.

[0154] A range of eukaryotic host systems may be utilized to investigate the activity of the mammalian desaturase control regions. Examples of hosts include, but are not limited to, fungi such as yeasts (*Saccharomyces cerevisiae*, and methylotrophic yeast such as *Pichia pastoris, Hansenula polymorpha, Kluyveromyces lactis* and *Schizosaccharomyces pombe*); mammalian cells, such as HepG2, HeLa, BHK, HEK-293, CHO, African Green Monkey kidney cells (e.g., COS 1, BSC1, BSC40, and BMT10), insect cells (e.g., Sf9), and human cells in tissue culture.

[0155] According to the present invention, the preferred cell system used in analysing control regions which are involved in the regulation of the level of mammalian desaturase gene expression is the mammalian cell line ZR-75-1 (ATCC # CRL 1500) or HepG2 (ATCC # HB-8065).

[0156] The control region-reporter vector, according to the present invention, can be constructed using conventional molecular biology, microbiology, and recombinant DNA techniques well known to those of skill in the art. Such techniques are explained fully in the literature, including Sambrook et al., 1989, *Molecular Cloning, A Laboratory Manual,* 2nd *edition*, Cold Spring Harbour Press, Cold Spring Harbour, N.Y. and Ausubel et al., 1994-, *Current Protocols in Molecular Biology*, John Wiley & Sons, New York, N.Y.

[0157] The practice of using a reporter gene to analyse nucleotide sequences which regulate transcription of genes involved in PUFA metabolism is well documented (Water K. M., 1997, *Biochim. Biophys. Acta.,* 1349: 33-42). Generally, a reporter gene encodes a polypeptide not otherwise produced by the host cell and which is detectable by analysis of the host cell. The product of a reporter gene is used to assess regulation of transcription via a control region/oligonucleotide sequence of the present invention. The expression of the reporter gene results in the formation of a reporter product (e.g. protein) which is readily detectable and hence, has a utility in its quantitative and/or qualitative capability to demonstrate that transcriptional activation has occurred. The reporter gene will be selected such that the reporter product will have physical and chemical characteristics, which facilitate its identification or detection, by means well known in the art. Reporter genes which are widely utilized in such studies include, but are not limited to, enzymes such as luciferase, chloramphenicol acetyl transferase (CAT), beta-galactosidase, esterases, phosphatases, proteases and other proteins such as green fluorescence protein (GFP) and human growth hormone. In a preferred embodiment, the reporter gene is CAT which will be detected through the level of specific enzymatic activity, which in turn correlates to the amount of enzyme that was made and hence, the level of expression of the reporter gene.

[0158] A reporter vector construct of the present invention includes essential elements for its propagation, selection and expression in either prokaryotic or eukaryotic cells.

[0159] The reporter vector of the present invention, which includes essential elements for its operability in prokaryotic or eukaryotic cells, is, preferably, pCAT-3-Enhancer or pGL3-Basic (Promega Corp., WI). The mammalian desaturase control region, derived from genomic DNA, is ligated by conventional methods in proper orientation (5' to 3') adjacent (5') to the start codon of the reporter gene with or without additional control elements. The region 3' to the coding sequence for the reporter gene will contain a transcription termination and polyadenylation site, for example, the SV40 polyA site. The desaturase control region and reporter gene, which are operably linked in the reporter vector, are transformed into a cloning host, preferably *E. coli*. The host is cultured and the replicated vector recovered in order to prepare sufficient quantities of the recombinant construction for subsequent transfection into a second host, preferably the mammalian cell line ZR-75-1 or HepG2.

[0160] Alternatively, an in vitro expression system can be accomplished, for example, by placing the nucleic acid sequence for a mammalian control region, described above, in an appropriate reporter vector designed for in vitro use. In vitro transcription can be carried out by adding nuclear extract from mammalian cells and other necessary reagents. Such in vitro reporter vectors may provide some or all of the expression signals necessary in the system used. These methods are well known in the art.

[0161] Accordingly, a vector construct of the present invention includes essential elements for its proliferation and selection in both eukaryotic and prokaryotic cells. Expression vectors of the invention include pYES2 and pYES2/CT (Invitrogen) which essentially comprise an origin of replication, an inducible promoter and two selectable marker genes. In particular, the pYES2/CT vector also contains a short DNA sequence that encodes for tags (e.g. V5/6×His epitopes) which allow the translated product, a tagged desaturase protein, to be easily identified and/or purified using commercially available antibodies and/or affinity chromatography columns. The pYES2 and pYES2/CT vectors, confer uracil prototrophy for selection in yeast, and a GAL1 galactose-inducible promoter for expression which is activated in the presence of galactose and situated upstream of the cloning site. Galactose-inducible promoters (GAL1, GAL7, and GAL 10) have been extensively utilized for high level and regulated expression of proteins in yeast (Lue et al., 1987, *Mol. Cell. Biol,* 7: 3446-3451 and Johnston M., 1987, *Microbiol Rev.,* 51: 458-476). Transcription from the GAL promoters is activated by the GAL4 protein, which binds to the promoter region and activates transcription when galactose is present In the absence of galactose, the antagonist GAL80 binds to GAL4 and prevents GAL4 from activating transcription. Addition of galactose prevents GAL80 from inhibiting activation by GAL4.

[0162] An expression vector may comprise a translation initiation or termination (e.g. stop) sequence oriented and operatively associated with the cDNA sequence encoding the mammalian desaturase (i.e. positioned upstream at the 5'-end or downstream at the 3'-end of the desaturase coding sequence, respectively). However, the translation initiation and termination codons may be already provided within the forward and reverse primer sequences, respectively, which are used to facilitate cloning of the mammalian desaturase genes into the pYES2 vector (see Example 5). Forward and reverse primers for cloning into pYES2/CT are designed to express a desaturase-V5/6×His tagged protein (see Example 5).

[0163] Thus, according to one aspect of the invention, there is provided a recombinant nucleic acid construct which contains a portion of a mammalian desaturase gene comprising the amino acid coding region and which has a heterologous promoter capable of initiating transcription of a fatty acid desaturase gene. In preferred embodiments of the invention, the amino acid coding region is derived from a human or a rat desaturase gene. In particular, the invention provides a nucleic acid construct having a promoter region which is preferably induced, a nucleic acid sequence encoding a functional mammalian (e.g. human or rat) fatty acid desaturase and a termination region, whereby the promoter region is operably associated with the nucleic acid sequence so as to effectively control expression of the nucleic acid sequence. Alternatively, the recombinant nucleic acid construct may comprise a heterologous transcriptional termination region functional in a host system. The recombinant nucleic acid construct is cloned as part of an expression vector, which can then be inserted into a host system.

[0164] In another embodiment of the invention, a polynucleotide encoding a mammalian (e.g. human or rat) fatty

acid desaturase may be ligated to a heterologous sequence to encode a tagged protein. For example, for screening of host systems for proteins exhibiting fatty acid desaturase activity, it may be useful to encode a tagged desaturase protein that is recognized by a commercially available antibody. A tagged protein may also be engineered to contain a cleavage site located between a desaturase coding sequence and the heterologous protein sequence, so that the fatty acid desaturase may be cleaved and purified away from the heterologous moiety.

[0165] Another aspect of the present invention is directed to a recombinant nucleic acid construct containing a control region of a mammalian fatty acid desaturase gene and a reporter gene. In preferred embodiments of the invention, the control region is derived from a human or a rat desaturase gene. The control region and the reporter sequence are operably linked so that the control region can effectively initiate, terminate or regulate the transcription or translation of the reporter sequence. The recombinant nucleic acid construct is cloned as part of an expression vector, which can then be inserted into a host system.

[0166] Host Systems

[0167] The invention provides a recombinant nucleic acid construct which contains a portion of a mammalian D6D gene including the amino acid coding region and which has a heterologous promoter capable of initiating transcription of a fatty acid desaturase gene. The amino acid coding region is derived from a human D6D gene. In particular, the invention provides a nucleic acid construct having a heterologous promoter region which is preferably induced, a nucleic acid sequence encoding a functional mammalian (e.g. human or rat) D6D and a termination region, whereby the promoter region is operably associated with the nucleic acid sequence so as to effectively control expression of the nucleic acid sequence. Alternatively, the recombinant nucleic acid construct may comprise a heterologous transcriptional termination region functional in a host system. The recombinant nucleic acid construct is cloned as part of an expression vector, which can then be inserted into a host system.

[0168] A polynucleotide encoding a mammalian (e.g. human or rat) D6D gene may be ligated to a heterologous sequence to encode a tagged protein. For screening of host systems that express D6D, it may be useful to encode a tagged desaturase protein that is recognized by a commercially available antibody. A tagged protein may also be engineered to contain a cleavage site located between a D6D coding sequence and the heterologous protein sequence, so that the fatty acid desaturase is cleaved and purified from the heterologous moiety.

[0169] Another aspect of the present invention is directed to a recombinant nucleic acid construct containing a control region of a mammalian D6D gene and a reporter gene. The control region is derived from a human D6D gene. The control region and the reporter sequence are operably linked so that the control region effectively initiates, terminates or regulates the transcription or translation of the reporter sequence. The recombinant nucleic acid construct is cloned as part of an expression vector, which is then inserted into a host system.

[0170] Accordingly, the host system is transformed/transfected by the nucleic acid construct containing the nucleic acid sequence of the D6D gene such that the promoter region and the termination region are operable and can, therefore, be used to achieve high level expression of a functionally active desaturase enzyme. A test component, which increases or decreases desaturase enzyme activity, is an enhancer or inhibitor, respectively. Consequently, defined test components can be used as a basis for the formulation or innovation of therapeutic agents to treat disease related to the level of active and regulated D6D enzyme in tissue.

[0171] The tansformed/transfected host cell is identified by selection for a marker gene contained on the introduced vector construct. The introduced marker gene, therefore, may confer antibiotic resistance, or encode an essential growth factor or enzyme, and permit growth on selective media when expressed in the transformed/transfected host. Typically, transformed/transfected hosts are selected due to their ability to grow on selective media. Selective media may contain an antibiotic or lack an essential growth nutrient necessary for the growth of the untransformed/untransfected host. Transformation of *Escherichia coli* cells and yeast cells was determined through selection on ampicillin-containing medium and uracil-deficient medium, respectively, based on the selection marker genes (e.g. beta-lactamase and URA3) present in the pYES2 and pYES2/CT vectors.

[0172] A cell-free expression system is achieved by placing the nucleic acid construct, comprising the coding sequence for a functional mammalian desaturase described above, into an appropriate expression vector designed for in vitro use and carrying out in vitro transcription/translation in a cell lysate, such as mRNA-dependent rabbit reticulocyte lysate. If required, additional components may be incorporated into the system such as essential co-factors and amino acids. Microsomal systems have been used successfully for testing enzyme activity from a number of different sources such as animal organs including liver, brain, heart, etc. and micro-organisms including yeast (de Antueno et al., 1994, *Lipids,* 29: 327-331; Todd et al., 1999, *Plant J.,* 17: 119-130; and Nishi et al., 2000, *Biochim. Biophys. Acta,* 1490: 106-108).

[0173] A microsomal host system is achieved by transforming/transfecting the host system with the nucleic acid construct containing the coding sequence for a functional mammalian desaturase described above, and isolating microsomes (Ausubel et al., 1994-, *Current Protocols in Molecular Biology*, John Wiley & Sons, New York, N.Y.). In vitro transcription/translation is carried out by adding rabbit reticulocyte lysate and essential cofactors; labelled amino acids can be incorporated if desired. Such in vitro expression vectors may provide some or all of the expression signals necessary in the system used. These methods are well known in the art and the components of the system are commercially available. The reaction mix is assayed directly for the polypeptide, for example by determining its specific enzymatic activity, or the synthesized polypeptide purified and then assayed for its specific enzymatic activity.

[0174] A cell system used in analyzing control regions which are involved in the regulation of the level of mammalian D6D gene expression is the mammalian cell lines ZR-75-1 (ATCC No. CRL-1500) or HepG2 (ATCC No. HB-8065).

[0175] Reporter genes which are widely utilized in such studies include, but are not limited to, enzymes such as

luciferase, chloramphenicol acetyl transferase (CAT), beta-galactosidase, esterases, phosphatases, proteases and other proteins such as green fluorescence protein (GFP) and human growth hormone. In preferred embodiments, the reporter gene is either CAT or luciferase which will be detected through the level of specific enzymatic activity, which in turn correlates to the amount of enzyme that was made and hence, the level of expression of the reporter gene.

[0176] A reporter vector of the present invention, which includes essential elements for its operability in prokaryotic or eukaryotic cells, is pCAT-3-Basic (Promega Corp., WI). The mammalian desaturase control region, derived from genomic DNA, is ligated by conventional methods in proper orientation (5' to 3') adjacent (5') to the start codon of the reporter gene with or without additional control elements. The region 3' to the coding sequence for the reporter gene contains a transcription termination and polyadenylation site, for example, the SV40 polyA site. The desaturase control region and reporter gene, which are operably linked in the reporter vector, are transformed into a cloning host, preferably *E. coli*. The host is cultured and the replicated vector recovered in order to prepare sufficient quantities of the recombinant construction for subsequent transfection into a second host, preferably the mammalian cell lines ZR-75-1 or HepG2.

[0177] Drug Screening Assays

[0178] When a preferred host cell is transfected or transformed with a DNA construct according to the present invention, it can be utilized in assays to identify potential test components that can modulate desaturase enzyme activity or alter the level of desaturase gene transcription via regulatory elements/oligonucleotide sequences. The screening assay typically is conducted by (1) growing the host cells transformed or transfected with desaturase genes or control regions to a suitable state of confluency in appropriate plates or flasks (e.g., microtiter wells, Erlenmeyers, etc.), (2) adding the test components to a series of wells or flasks, and (3) determining the signal level (e.g. desaturase activity or level of gene expression) after an incubation period that is suitable to demonstrate a measurable signal in the assay system chosen. The wells or flasks, containing varying proportions and/or classes of test components can be evaluated by signal activation within the treated cells. Candidates that demonstrate modulation of desaturase enzyme activity or reporter gene expression are then selected for further evaluation as clinical therapeutic agents.

[0179] A host cell strain may be chosen for its ability to modulate the expression of the inserted sequences, or to process the gene product in the desired fashion. Such modifications (e.g. glycosylation) and processing (e.g. cleavage) of protein products may be important for the function of the protein. Different host cells have characteristic and specific mechanisms for the post-translational processing and modification of proteins and gene products which may also be important to ensure correct processing and functioning of the expressed foreign protein. Appropriate cell lines or host systems can be chosen to ensure the correct modification and processing of the foreign protein expressed. To this end, prokaryotic or eukaryotic host cells, which possess the cellular machinery for proper processing of the primary transcript, and for proper glycosylation, phosphorylation and folding of the gene product may be used. Such prokaryotic, or eukaryotic host cells include but are not limited to *E. coli, Bacillus subtilis, Pseudomonas putida, Saccharomyces cerevisiae, Schizosaccharomyces pombe, Pichia pastoris, Hansenula polymorpha, Kluyveromyces lactis*, ZR-75-1, Chang, CHO, VERO, BHK, HeLa, COS, MDCK, 293, 3T3, WI38, and U937 cells.

[0180] In a preferred embodiment of the present invention, the medium for conducting the drug screening method is an eukaryotic cell, including fungal and mammalian cells.

[0181] Modulation of Mammalian Desaturase Activity

[0182] More specifically, an embodiment of the present invention relates to a drug screening assay using transformed yeast as whole cells, spheroplasts, cell homogenates or organelles (e.g. microsomes, etc.) to identify candidate agents that modulate the enzymatic activity of a mammalian desaturase. In a preferred embodiment of the present invention the host yeast *Saccharomyces cerevisiae*, strain INVSc1, Invitrogen San Diego, Calif.) is transformed with the yeast expression vector, pYES2 (Invitrogen), containing the mammalian desaturase coding sequence. Yeast cells are selected for use in the present method because (1) they have not shown fatty acid delta-6-desaturase activity, (Aki et al, 1999), (2) the transcription and translation processes are similar, if not identical, to processes that occur in mammalian cells, and (3) yeast cells are often more amenable to genetic manipulation than mammalian cells, and they grow much more rapidly (Guthrie C. and Fink G., 1991, *Methods in Enzymology*, 194). Thus, yeast cells provide an excellent model for eukaryotic gene expression and for studying the modulation of mammalian desaturase activity.

[0183] When a preferred host cell, such as a yeast cell, is transformed with a DNA construct according to the present invention, it can be utilized in assays to identify potential test components that can modulate desaturase activity. Test components having the potential to modulate desaturase activity can be identified by (1) contacting the transformed host cell with the test component for a fixed period of time, and (2) determining the level of lipid metabolite (e.g. the level of product produced from substrate) within the treated cells. This level of metabolite in one cell can then be compared to the level of metabolite in the absence of the test component. The difference between the levels of metabolite, if any, indicates whether the test component of interest modulates desaturase activity. Furthermore, the magnitude of the level of lipid metabolite generated between the treated and untreated cells provides a relative indication of the strength of that compound(s) as a modulator of desaturase activity. Rat liver microsomes (obtained as described in other Examples) may be used in conjunction with the preferred host system to corroborate the strength of that compound(s) as a modulator of desaturase activity.

[0184] Modulation of Mammalian Desaturase Gene Expression

[0185] The present invention also relates to a drug screening assay using mammalian cells as host systems to observe the regulation of desaturase gene expression and identify test components that modulate the expression of a reporter gene driven by desaturase gene control regions or regulatory elements. In a preferred embodiment of the present invention, the ZR-75-1 (human mammary carcinoma) cell line is used as the host system which is transfected with the reporter

vector, pCAT-3-Enhancer (chloramphenicol acetyl transferase; Promega Corp., WI) containing the mammalian desaturase control sequence. ZR-75-1 cells were selected for use in the present method because (1) this cell line shows high level of delta-6-desaturase mRNA expression (as shown by Northern blot), and (2) it is amenable for transfection. Alternatively, the HepG2 cell line was used as the host system for stable transfection of the hD6D control region inserted in the pGL3-Basic reporter vector (luciferase; Promega Corp., WI). This human hepatoma cell line was chosen to study regulation of the D6D promoter activity because of previous gene expression results (Northern blots) which indicate regulation of the D6D gene in this cell line that is similar to what was seen in rat liver.

[0186] When a preferred host cell line, such as ZR-75-1 or HepG2, is transfected with a reporter DNA construct according to the present invention, it can be utilized in assays to identify potential test components that can modulate the level of gene transcription via functionally active regulatory elements/oligonucleotide sequences. Test components having the potential to alter the level of gene transcription can be identified by (1) contacting the transfected host cell with the test component for a fixed period of time, and (2) determining the level of gene expression (e.g. the level of CAT produced) within the treated cells. This expression level can then be compared to the expression level of the reporter gene in the absence of the compound(s). The difference between the levels of gene expression, if any, indicates whether the compound(s) of interest modifies the functionality of the DNA regulatory elements. Furthermore, the magnitude of the level of reporter product expressed between the treated and untreated cells provides a relative indication of the strength of that compound(s) as a modulator of the desaturase gene transcription via transcriptional DNA regulatory elements.

[0187] Host Systems and Drug Screening

[0188] The invention includes methods for screening nucleotides, proteins, compounds or pharmacological agents, which enhance or inhabit D6D gene expression at the transcriptional level or modulate the D6D activity. To this end, cell-based, cell lysate and/or purified enzyme assays are used to detect these enhancing or inhibiting components.

[0189] D6D gene expression has been associated with diabetes and related disorders, arterial hypertension; hypercholesterolemia; atherosclerotic heart disease; chronic inflammatory disorders; autoimmune disorders; allergic eczema and other atopic disorders; inflammatory processes such as rheumatoid arthritis; diminished lymphocyte proliferation, T-cell-mediated cytotoxicity, natural killer cell activity, macrophage-mediated cytotoxicity, monocyte and neutrophil chemotaxis, major histocompatibility class II expression and antigen presentation, production of pro-inflammatory cytokines (interleukins 1 and 6, tumour necrosis factor) and adhesion molecule expression; eczema; psoriasis; acute respiratory distress syndrome (ARDS); articular cartilage degradation (ACD); and cancer.

[0190] A present inventors' human diabetic clinical trial has provided data indicating that AA and EPA were reduced in the plasma and red cell phospholipids of Type 1 diabetics. This study supports and expands a multi-center clinical trial sponsored by Scotia Pharmaceuticals in which enteral administration of n-6 PUFAs ameliorates neurophysiological parameters of mild diabetic neuropathy (Keen et al., 1993, *Diabetes Care,* 16: 8-15). Reduced levels of long chain n-6 fatty acids have been reported (Arisaka et al., 1986, *J. Paediatr. Gastroenterol. Nutr.,* 5: 878-882; Tilvis R. S. and Miettinen T. A., 1985, *J. Clin. Endocrinol. Metab.,* 61: 741-745; and van Doormaal et al., 1988, *Diabetologia,* 31: 576-584). The level of DGLA was not reduced in the Type 1 diabetic group, indicating that the reduction of AA may be due to reduced delta-6-desaturase activity.

[0191] In a present inventors' diabetic rat study, the plasma phospholipid AA content was reduced 31% and 27% in the 2 week and 7 week streptozotocin-induced diabetic rats, respectively. As in the human diabetic study, the DGLA levels remained unchanged compared to controls, so the reduced levels of AA and EPA were consistent with a detected reduction in delta-6-desaturase activity. Reduced activity of the desaturase system in diabetes was first reported by Brenner et al., 1968, *Am. J. Physiol.,* 215: 63-70. Subsequently, this finding has been verified (Mimouni V. and Poisson J. P., 1992, *Biochim. Biophys. Acta,* 1123: 296-302; Dang et al., 1989, *Lipids,* 24: 882-889; and Faas F. H. and Carter W. J., 1980, *Lipids,* 15: 953-961) and is considered to be a key factor in the development of secondary complications of diabetes. In the streptozotocin diabetic rat study, it was determined that the delta-6-desaturase activity in hepatic microsomes from diabetic rats was reduced by 37% compared to the control rats. These findings support the hypothesis that delta-6-desaturase is a potential drug target in diabetes and also a useful lipid metabolic compound for drug screening assays.

[0192] The present invention features a drug screening method for identifying nucleotides, proteins, compounds, and/or pharmacological agents which modulate or regulate the transcription of a mammalian D6D gene. This method includes (1) providing a novel nucleic acid construct having a control region of a mammalian desaturase gene and a heterologous nucleic acid sequence (e.g. a reporter gene), wherein the control region is operably associated with the nucleic acid sequence so that it effectively initiates, terminates or regulates the transcription of the nucleic acid sequence, all of which are introduced into a cell or cell lysate using an expression vector containing the novel nucleic acid construct, (2) contacting the cell or cell lysate with a test component, (3) determining whether the test component is capable of altering the level of transcription of the nucleic acid sequence, and (4) selecting those components which exhibit such activity. In this regard, the defined test components can be used as a basis for the formulation or innovation of therapeutic drugs to treat disease related to the level of D6D gene expression. Test components, which increase or decrease the level of transcription of the reporter sequence, are enhancers or inhibitors, respectively.

[0193] In particular, the present invention embodies a method for the identification of useful and functional portions of the D6D control region and various functional and regulatory elements within the control region, which are associated with the level of expression of the desaturase gene. Functional portions of the desaturase control region which result in altered levels of gene expression are determined through the manipulation (e.g. deletion, site-directed mutagenesis, etc.) of various segments of the region, as well as through the direct or indirect effect of modulators.

[0194] The host system for conducting the drug screening method can be eukaryotic cells, including fungal or mammalian cells. More specifically, an embodiment of the present invention relates to a drug screening assay using transformed yeast as whole cells, spheroplasts, cell homogenates, organelles (e.g. microsomes, etc.) or purified enzyme to identify candidate agents that modulate the enzymatic activity of a mammalian D6D. In an embodiment of the present invention the host yeast *Saccharomyces cerevisiae*, strain INVSc1 (Invitrogen, CA), is transformed with the yeast expression vectors, pYES2 or pYES2/CT (Invitrogen), containing the mammalian D6D coding sequence. Yeast cells are selected for use in the present method because (1) they have not shown fatty acid delta-6-desaturase activity (Aki et al, 1999, *Biochem. Biophys. Res. Commun.*, 255: 575-579), (2) their transcription and translation processes are similar, if not identical, to processes that occur in mammalian cells, and (3) they are often more amenable to genetic manipulation than mammalian cells, and they grow much more rapidly (Guthrie C. and Fink G., 1991, *Meth. Enzymol*, 194). Thus, yeast cells provide an excellent model for eukaryotic gene expression and for studying the modulation of mammalian D6D activity.

[0195] When a host cell, such as a yeast cell, is transformed with a DNA construct according to the present invention, it is utilized in assays to identify test components that modulate desaturase activity. Test components that modulate D6D activity are identified by (1) contacting the transformed host cell with the test component for a fixed period of time, and (2) determining the level of lipid metabolite (i.e. the level of product produced from substrate) or associated cofactors within the treated cells. This level of metabolite in one cell can then be compared to the level of metabolite in the absence of the test component. The difference between the levels of metabolite, if any, indicates whether the test component of interest modulates D6D activity. Furthermore, the magnitude of the level of lipid metabolite generated between the treated and untreated cells provides a relative indication of the strength of that compound(s) as a modulator of desaturase activity. Rat liver microsomes are used in conjunction with the preferred host system to corroborate the strength of that compound(s) as a modulator of desaturase activity.

[0196] A drug screening assay is also carried out using mammalian cells as host systems to observe the regulation of D6D gene expression and identity test components that modulate the expression of a reporter gene driven by D6D gene control regions or regulatory elements. ZR-75-1 or HepG2 cell lines are preferably used as the host systems, which are transfected with the reporter vectors, pCAT-3-Basic (Promega) or pGL3-Basic (Promega) containing the mammalian D6D control sequence.

[0197] When a preferred host cell line, such as ZR-75-1, is transfected with a reporter DNA construct according to the present invention, it is utilized in assays to identify test components that modulate the level of gene transcription via functionally active regulatory elements/oligonucleotide sequences.

[0198] Test components that alter the level of gene transcription can be identified by (1) contacting the transfected host cell with the test component for a fixed period of time, and (2) determining the level of gene expression (e.g. CAT activity) within the treated cells. This expression level is compared to that of the reporter gene in the absence of the compound(s). The difference between the levels of gene expression, if any, indicates whether the compound(s) of interest modifies the functionality of the DNA regulatory elements. Furthermore, the magnitude of the level of reporter product expressed between the treated and untreated cells provides a relative indication of the strength of that compound(s) as a modulator of the D6D gene transcription via transcriptional DNA regulatory elements.

[0199] In an embodiment, a high-throughput screening protocol is used to survey a large number of test compounds for their ability to modulate or regulate the transcription of a mammalian D6D gene through their effect on the desaturase control region. Accordingly, the design of the transcriptional system makes it possible to screen a large selection of components as potential therapeutic agents that alter D6D gene expression thereby increasing or decreasing tissue levels of a functional D6D enzyme, the physiological significance of which includes the normalization of lipid metabolites.

[0200] For the drug screening methods described herein, the host system may be a cell, tissue, organ, organism or any part thereof, which provides an environment or conditions that allow for, or enable, transcription and/or transcription followed by subsequent translation to yield a functional protein or polypeptide. Organisms would include animals such as mammals. In an embodiment of the invention, the drug screening methods are conducted in prokaryotic and eukaryotic cells. In embodiments of the invention, the eukaryotic cells include yeast cells and mammalian cells.

[0201] Potential antagonists include small organic molecules, peptides, polypeptides and antibodies that bind to a polynucleotide or polypeptide of the invention and thereby inhibit or extinguish its activity. Potential antagonists also may be small organic molecules, a peptide, a polypeptide such as a closely related protein or antibody that bind the same sites on a binding molecule, such as a binding molecule, without inducing delta-6-desaturase-induced activities, thereby preventing the action of delta-6-desaturase by interfering with substrate binding.

[0202] Potential antagonists include a small molecule, which bind to and occupy the binding site of the polypeptide thereby preventing binding to cellular binding molecules, such that normal biological activity is prevented. Examples of small molecules include but are not limited to small organic molecules, peptides or peptide-like molecules. Other potential antagonists include antisense molecules (see Okano et al., 1988, EMBO J., 7: 3407-3412 for a description of these molecules).

[0203] Selective modulators may include, for example, antibodies and other proteins or peptides which specifically bind to the delta-6-desaturase or delta-6-desaturase nucleic acid, oligonucleotides which specifically bind to delta-6-desaturase (see Patent Cooperation Treaty International Publication No. WO93/05182 published Mar. 18, 1993) which describes methods for selecting oligonucleotides which selectively bind to target biomolecules) or delta-6-desaturase nucleic acid (e.g. antisense oligonucleotides) and other non-peptide natural or synthetic compounds which specifically bind to the delta-6-desaturase or delta-6-desaturase nucleic acid.

[0204] Targets for the development of selective modulators include, for example: (1) the regions of the delta-6-desaturase which contact other proteins and/or localize the delta-6-desaturase within a cell and (2) the regions of the delta-6-desaturase which bind substrate.

[0205] Thus, according to another aspect of the invention there is provided a drug screening method for identifying nucleotides, proteins, compounds and/or pharmacological agents that effectively modulate the activity of fatty acid desaturase enzymes and hence, fatty acid profiles. The method comprises (1) producing a nucleic acid construct having a promoter region, which is preferably induced, a nucleic acid sequence encoding a functional fatty acid desaturase enzyme, whereby the promoter region is operably associated with the nucleic acid sequence, and a termination sequence, all of which are introduced into a cell or cell lysate using an expression vector containing the nucleic acid construct, (2) contacting the cell or cell lysate with a test component, (3) evaluating the enzymatic activity of a desaturase polypeptide encoded by the nucleic acid sequence by assaying for a measurable difference in the level of lipid metabolite as an indicator of the ability of the test component to modulate fatty acid desaturase enzyme activity, and (4) selecting those components which exhibit such activity. The known substrate for the fatty acid desaturase may optionally be exogenously supplied to the cell or cell lysate.

[0206] Accordingly, the host system is transformed/transfected by the nucleic acid construct containing the nucleic acid sequence of the fatty acid desaturase gene such that the promoter region and the termination region are operable and can, therefore, be used to achieve high level expression of a functionally active desaturase enzyme. A test component which increases or decreases desaturase enzyme activity is an enhancer or inhibitor, respectively. Consequently, defined test components can be used as a basis for the formulation or innovation of therapeutic agents to treat disease related to the level of active and regulated fatty acid desaturase enzymes in tissue.

[0207] A microsomal host system may be achieved by transforming/transfecting the host system with the nucleic acid construct containing the coding sequence for a functional mammalian desaturase described above, and isolating microsomes (Ausubel et al., 1994-, *Current Protocols in Molecular Biology*, John Wiley & Sons, New York, N.Y.).

[0208] A cell-free expression system may be achieved by placing the nucleic acid construct comprising the coding sequence for a functional mammalian desaturase described above, inserting it into an appropriate expression vector designed for it vitro use and carrying out in vitro transcription/translation in a cell lysate, such as mRNA-dependent rabbit reticulocyte lysate. If required, additional components may be incorporated into the system such as essential co-factors and amino acids.

[0209] In a preferred embodiment, a high-throughput screening protocol is used to survey a large number of test compounds for their ability to modulate the enzymatic activity of a mammalian fatty acid desaturase. Accordingly, the design of the drug screening method makes it possible to screen a large selection of components as potential therapeutic agents that alter fatty acid desaturase activity thereby increasing or decreasing levels of specific lipid metabolites, the physiological significance of which includes the normalization of lipid metabolism.

[0210] In another aspect, the present invention features a drug screening method for identifying nucleotides, proteins, compounds, and/or pharmacological agents which modulate or regulate the transcription of a mammalian fatty acid desaturase gene. This method includes (a) providing a novel nucleic acid construct having a control region of a mammalian desaturase gene and a heterologous nucleic acid sequence (e.g. a reporter gene), wherein the control region is operably associated with the nucleic acid sequence so that it can effectively initiate, terminate or regulate the transcription of the nucleic acid sequence, all of which are introduced into a cell or cell lysate using an expression vector containing the novel nucleic acid construct, (b) contacting the cell or cell lysate with a test component, (c) determining whether the test component is capable of altering the level of transcription of the nucleic acid sequence, and (d) selecting those components which exhibit such activity. In this regard, the defined test components can be used as a basis for the formulation or innovation of therapeutic drugs to treat disease related to the level of fatty acid desaturase gene expression. Test components, which increase or decrease the level of transcription of the reporter sequence, are enhancers or inhibitors, respectively.

[0211] In particular, the present invention embodies a method for the identification of useful and functional portions of the fatty acid desaturase control region and various functional and regulatory elements within the control region which are associated with the level of expression of the desaturase gene. Functional portions of the desaturase control region which result in altered levels of gene expression are determined through the manipulation (e.g. deletion, site-directed mutagenesis, etc.) of various segments of the region, as well as through the direct or indirect effect of modulators.

[0212] A cell-free expression system may be achieved by placing the novel nucleic acid construct comprising the control region of a mammalian desaturase gene and a reporter sequence as described above, inserting it into an appropriate expression vector designed for in vitro use and carrying out in vitro expression in a cell lysate. If required, additional components may be incorporated into the system such as essential co-factors and other reagents.

[0213] In a preferred embodiment, a high-throughput screening protocol is used to survey a large number of test compounds for their ability to modulate or regulate the transcription of a mammalian fatty acid desaturase gene through their effect on the desaturase control region. Accordingly, the design of the transcriptional system makes it possible to screen a large selection of components as potential therapeutic agents that alter fatty acid desaturase gene expression thereby increasing or decreasing tissue levels of a functional desaturase enzyme, the physiological significance of which includes the normalization of lipid metabolites.

[0214] For the drug screening methods described above, the host system may be a cell, tissue, organ, organism or any part thereof, which provides an environment or conditions that allow for, or enable, transcription and/or transcription followed by subsequent translation to yield a functional

protein or polypeptide. Organisms would include animals such as mammals. In a preferred embodiment of the invention, the drug screening methods are conducted in prokaryotic and eukaryotic cells. In preferred embodiments of the invention, the eukaryotic cells include yeast cells and mammalian cells.

[0215] Drug Design

[0216] Modulation of delta-6-desaturase gene function can be accomplished by the use of therapeutic agents or drugs which can be designed to interact with different aspects of delta-6-desaturase control region structure or function. For example, a drug or antibody can bind to a structural fold of the control region to correct a defective structure. Alternatively, a drug might bind to a specific functional residue and increase its affinity for a substrate or cofactor. Efficacy of a drug or agent can be identified by a screening program in which modulation is monitored in vitro in cell systems in which a delta-6-desaturase gene protein is expressed. Alternatively, drugs can be designed to modulate delta-6-desaturase gene activity from knowledge of the structure and function correlations and from knowledge of the specific defect in the various NF1 mutant proteins (see Copsey D. N. and Delnatte S. Y. J., 1988, *Genetically Engineered Human Therapeutic Drugs*, Stockton Press, New York).

[0217] Gene Therapy

[0218] A variety of gene therapy approaches may be used in accordance with the invention to modulate expression of delta-6-desaturase in vivo. For example, antisense DNA molecules may be engineered and used to block delta-6-desaturase DNA in vivo. In another alternative, oligonucleotides designed to hybridize to the 5' region of the delta-6-desaturase control sequence and form triple helix structures may be used to block or reduce transcription of the delta-6-desaturase. In yet another alternative, nucleic acid encoding the full length wild-type delta-6-desaturase control region may be introduced in vivo into cells which otherwise would be unable to produce the wild-type delta-6-desaturase product in sufficient quantities or at all.

[0219] For example, in conventional replacement therapy, gene product or its functional equivalent is provided to the patient in therapeutically effective amounts. Delta-6-desaturase protein can be purified using conventional techniques such as those described in Deutcher, M. (editor), 1990, *Guide to Protein Purification. Meth.Enzymol.:* 182. Sufficient amounts of gene product or protein for treatment can be obtained, for example, through cultured cell systems or synthetic manufacture. Drug therapies which stimulate or replace the gene product can also be employed. Delivery vehicles and schemes can be specifically tailored to the particular protein or drug being administered.

[0220] Gene therapy using recombinant technology to deliver the gene into the patient's cells or vectors, which will supply the patient with gene product in vivo, is also contemplated as within the scope of the present invention. Retroviruses have been considered a preferred vector for experiments in somatic gene therapy, with a high efficiency of infection and stable integration and expression (Orkin, et al., 1988, *Prog. Med. Genet.* 7: 130-142). For example, delta-6-desaturase cDNA can be cloned into a retroviral vector and driven from either its endogenous promoter of from the retroviral LTR (long terminal repeat). Other delivery systems which can be utilized include adeno-associated virus (AAV) (McLaughlin et al., 1988, *J. Virol* 62: 1963-1973), vaccinia virus (Moss et al., 1987, *Annu. Rev. Immunol.* 5: 305-324), bovine papilloma virus (Rasmussen, et al.,1987, *Meth. Enzymol.* 139: 642-654), or member of the herpesvirus group such as Epstein-Barr virus (Margolskee, et al., 1988, *Mol Cell. Biol.* 8: 2837-2847.

[0221] In another embodiment, the antisense, ribozyme and triple helix nucleotides are designed to inhibit the translation or transcription of delta-6-desaturase. To accomplish this, the oligonucleotides used should be designed on the basis of relevant sequences unique to delta-6-desaturase control region.

[0222] For example, and not by way of limitation, the oligonucleotides should not fall within those region where the nucleotide sequence of a subject polynucleotide is most homologous to that of other fatty acid enzyme polynucleotides, herein referred to as "unique regions".

[0223] In the case of antisense molecules, it is preferred that the sequence be chosen from the unique regions. It is also preferred that the sequence be at least 18 nucleotides in length in order to achieve sufficiently strong annealing to the target mRNA sequence to prevent translation of the sequence. Izant J. G. and Weintraub H., 1984, *Cell,* 36: 1007-1015; Rosenberg et al., 1985, *Nature,* 313: 703-706.

[0224] In the case of the "hammerhead" type of ribozymes, it is also preferred that the target sequences of the ribozymes be chosen from the unique regions. Ribozymes are RNA molecules which possess highly specific endoribonuclease activity. Hammerhead ribozymes comprise a hybridizing region which is complementary in nucleotide sequence to at least part of the target RNA, and a catalytic region which is adapted to cleave the target RNA. The hybridizing region contains nine or more nucleotides. Therefore, the hammerhead ribozymes of the present invention have a hybridizing region which is complementary to the sequences listed above and is at least nine nucleotides in length. The construction and production of such ribozymes is well known in the art and is described more fully in Haseloff J. and Gerlach W. L., 1988, *Nature,* 334: 585-591.

[0225] The ribozymes of the present invention also include RNA endoribonucleases (hereinafter "Cech-type ribozymes") such as the one which occurs naturally in Tetrahymena Thermophila (known as the IVS, or L-19 IVS RNA) and which has been extensively described by Thomas Cech and collaborators (Zaug et al., 1984, *Science,* 224: 574-578; Zaug A. J. and Cech T. R, 1986, *Science,* 231: 470-475; Zaug, et al., 1986, *Nature,* 324: 429-433; published International patent application No. WO 88/04300 by University Patents Inc. June, 1988; Been M. D. and Cech T. R, 1986, *Cell,* 47: 207-216). The Cech endoribonucleases have an eight base pair active site which hybridizes to a target RNA sequence whereafter cleavage of the target RNA takes place. The invention encompasses those Cech-type ribozymes which target eight base-pair active site sequences that are present in a subject polynucleotide but not other polynucleotides for fatty acid enzymes.

[0226] The compounds can be administered by a variety of methods which are known in the art including, but not limited to the use of liposomes as a delivery vehicle. Naked

DNA or RNA molecules may also be used where they are in a form which is resistant to degradation such as by modification of the ends, by the formation of circular molecules, or by the use of alternate bonds including phosphothionate and thiophosphoryl modified bonds. In addition, the delivery of nucleic acid may be by facilitated transport where the nucleic acid molecules are conjugated to poly-lysine or transferrin. Nucleic acid may also be transported into cells by any of the various viral carriers, including but not limited to, retrovirus, vaccinia, AAV, and adenovirus.

[0227] Alternatively, a recombinant nucleic acid molecule which encodes, or is, such antisense, ribozyme, triple helix, or subject polynucleotide molecule can be constructed. This nucleic acid molecule may be either RNA or DNA. If the nucleic acid encodes an RNA, it is preferred that the sequence be operatively attached to a regulatory element so that sufficient copies of the desired RNA product are produced. The regulatory element may permit either constitutive or regulated transcription of the sequence. In vivo, that is, within the cells or cells of an organism, a transfer vector such as a bacterial plasmid or viral RNA or DNA, encoding one or more of the RNAs, may be transfected into cells e.g. (Llewellyn et al., 1987, *J. Mol. Biol.*, 195: 115-123; Hanahan et al., 1983, *J. Mol. Biol.*, 166: 557-580). Once inside the cell, the transfer vector may replicate, and be transcribed by cellular polymerases to produce the RNA or it may be integrated into the genome of the host cell. Alternatively, a transfer vector containing sequences encoding one or more of the RNAs may be transfected into cells or introduced into cells by way of micromanipulation techniques such as microinjection, such that the transfer vector or a part thereof becomes integrated into the genome of the host cell.

[0228] Composition, Formulation, and Administration of Pharmaceutical Compositions

[0229] The pharmaceutical compositions of the present invention may be manufactured in a manner that is itself known, e.g., by means of conventional mixing, dissolving, granulating, dragee-making, levigating, emulsifying, encapsulating, entrapping or lyophilizing processes.

[0230] Pharmaceutical compositions for use in accordance with the present invention thus may be formulated in conventional manner using one or more physiologically acceptable carriers comprising excipients and auxiliaries which facilitate processing of the active compounds into preparations which can be used pharmaceutically. Proper formulation is dependent upon the route of administration chosen.

[0231] For injection, the agents of the invention may be formulated in aqueous solutions, preferably in physiologically compatible buffers such as Hanks's solution, Ringer's solution, or physiological saline buffer. For transmucosal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art.

[0232] For oral administration, the compounds can be formulated readily by combining the active compounds with pharmaceutically acceptable carriers well known in the art. Such carriers enable the compounds of the invention to be formulated as tablets, pills, dragees, capsules, liquids, gels, syrups, slurries, suspensions and the like, for oral ingestion by a patient to be treated. Pharmaceutical preparations for oral use can be obtained solid excipient, optionally grinding a resulting mixture, and processing the mixture of granules, after adding suitable auxiliaries, if desired, to obtain tablets or dragee cores. Suitable excipients are, in particular, fillers such as sugars, including lactose, sucrose, mannitol, or sorbitol; cellulose preparations such as, for example, maize starch, wheat starch, rice starch, potato starch, gelatin, gum tragacanth, methyl cellulose, hydroxypropylmethyl-cellulose, sodium carboxymethylcellulose, and/or polyvinylpyrrolidone (PVP). If desired, disintegrating agents may be added, such as the cross-linked polyvinyl pyrrolidone, agar, or alginic acid or a salt thereof such as sodium alginate.

[0233] Dragee cores are provided with suitable coatings. For this purpose, concentrated sugar solutions may be used, which may optionally contain gum arabic, talc, polyvinyl pyrrolidone, carbopol gel, polyethylene glycol, and/or titanium dioxide, lacquer solutions, and suitable organic solvents or solvent mixtures. Dyestuffs or pigments may be added to the tablets or dragee coatings for identification or to characterize different combinations of active compound doses.

[0234] Pharmaceutical preparations which can be used orally include push-fit capsules made of gelatin, as well as soft, sealed capsules made of gelatin and a plasticizer, such as glycerol or sorbitol. The push-fit capsules can contain the active ingredients in admixture with filler such as lactose, binders such as starches, and/or lubricants such as talc or magnesium stearate and, optionally, stabilizers. In soft capsules, the active compounds may be dissolved or suspended in suitable liquids, such as fatty oils, liquid paraffin, or liquid polyethylene glycols. In addition, stabilizers may be added. All formulations for oral administration should be in dosages suitable for such administration.

[0235] For buccal administration, the compositions may take the form of tablets or lozenges formulated in conventional manner.

[0236] For administration by inhalation, the compounds for use according to the present invention are conveniently delivered in the form of an aerosol spray presentation from pressurized packs or a nebulizer, with the use of a suitable propellant, e.g., dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane, carbon dioxide or other suitable gas. In the case of a pressurized aerosol the dosage unit may be determined by providing a valve to deliver a metered amount. Capsules and cartridges of e.g. gelatin for use in an inhaler or insufflator may be formulated containing a powder mix of the compound and a suitable powder base such as lactose or starch.

[0237] The compounds may be formulated for parenteral administration by injection, e.g., by bolus injection or continuous infusion. Formulations for injection may be presented in unit dosage form, e.g., in ampoules or in multidose containers, with an added preservative. The compositions may take such forms as suspensions, solutions or emulsions in oily or aqueous vehicles, and may contain formulatory agents such as suspending, stabilizing and/or dispersing agents.

[0238] Pharmaceutical formulations for parenteral administration include aqueous solutions of the active compounds in water-soluble form. Additionally, suspensions of the active compounds may be prepared as appropriate oily injection suspensions. Suitable lipophilic solvents or